THE ROLE OF BOVINE ADENOVIRUS (BAdV)-3 PROTEIN pVIII IN VIRUS REPLICATION

A Thesis
Submitted to the Faculty of Graduate Studies and Research
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy
in the
Department of Veterinary Microbiology
University of Saskatchewan
Saskatoon

By
Lisanework Eshetu Ayalew

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the libraries of this university may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, whole or in part, for scholarly purposes may be granted by the professors who supervised my thesis work or in their absence, the Head of the Department or the Dean of the college in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts therof for financial gain shall not be allowed without any written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Request for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Veterinary Microbiology

University of Saskatchewan,

Saskatoon, Saskatchewan,

S7N 5B4

ABSTRACT

Bovine adenovirus (BAdV)-3 is a non-enveloped icosahedral DNA virus, which replicates in the nucleus of infected cells, and is being developed as a vector for vaccination for humans and animals. The genome of BAdV-3 is organized into early, intermediate and late genes and it has thirty three predicted open reading frames (Reddy et al., 1998). The late region of BAdV-3 is divided into seven families (L1-L7) (Reddy et al., 1998). One of the proteins expressed in the L-6 region encodes a protein called pVIII, which is a minor capsid protein connecting the core with the inner surface of the capsid. The objective of the current study was to characterize pVIII protein of BAdV-3 and to examine its role in the life cycle of BAdV-3.

Anti-pVIII serum detected a protein of 24 kDa at 12-48 hr post infection and an additional protein of 8 kDa at 24-48 hr post infection. While a 24 kDa protein is detected in empty capsids, only the C-terminal cleaved protein of 8 kDa is detected in the mature virion suggesting that amino acids 147-216 of conserved C- terminus of BAdV-3 pVIII are incorporated in mature virions. The pVIII protein predominantly localizes to the nucleus of BAdV-3 infected cells utilizing the classical importin α /β dependent nuclear import pathway. Analysis of mutant pVIII demonstrated that amino acids 57-72 of the conserved N-terminus bind to importin α-3 with high affinity and are required for the nuclear localization. Detection of hexon associated with both, precursor (24 kDa) and cleaved (8 kDa) form of pVIII suggests that the C-terminus of pVIII interacts with Hexon.

Based on yeast II hybrid screening assay, we identified the cellular protein DDX3 as an interacting protein partner of pVIII. Earlier, targeting of DDX3 by few viral proteins has defined its role in mRNA transport (Yedavalli et al., 2004) and induction of interferon production (Schroder et al., 2008; Wang et al., 2009). Here, we provide evidence regarding the involvement

of DDX3 in cap dependent cellular mRNA translation and show that targeting of DDX3 by the adenovirus pVIII protein abolishes cap-dependent mRNA translation function of DDX3 in virus infected cells. Adenovirus late protein pVIII interacts with DDX3 in transfected and bovine adenovirus (BAdV-3) infected cells. pVIII inhibited capped mRNA translation *in-vitro* and *in-vivo* by limiting the amount of DDX3 and eIF3. Diminished amount of DDX3 and eIFs including eIF3, eIF4E and PABP were present in cap binding complex in BAdV-3 infected or pVIII transfected cells with no trace of pVIII in the cap binding complex. The total amount of eIFs appeared similar in uninfected or BAdV-3 infected cells. The co-immunoprecipitation experiments indicated the absence of direct interaction between pVIII and eIF3, eIF4E or PABP. These data indicate that interaction of pVIII with DDX3 depletes eIF3, eIF4E and PABP from the cap-binding complex. We conclude that DDX3 promotes cap-dependent cellular mRNA translation and BAdV-3 pVIII inhibits translation of capped cellular mRNA by excluding functional cap-binding complex from the capped cellular mRNA.

BAdV-3 infection of DDX3 positive cells significantly inhibits cellular protein synthesis at late times post-infection. Interestingly, knockdown of DDX3 resulted in significant reduction in virus yield and expression of BAdV-3 late proteins at late times post-infection. Our results suggest that selective translation of BAdV-3 late mRNAs observed at late time post-infection of DDX3 positive cells is abrogated in DDX3 knock down cells. Moreover, the reduction in the extent of protein synthesis is evidenced by less functional 80S and polysomes in pVIII expressing plasmid transfected cells. Alternatively, DDX3 and pVIII binds to BAdV-3 tripartite leader (TPL) and the translation of mRNAs containing TPL at their 5’ ends is enhanced in the presence of pVIII and DDX3 proteins. From this observation, we concluded that pVIII and DDX-3 might promote the translation of late viral mRNAs by interacting with TPL.

ACKNOWLEGEMENT

For the most part I would like to forward my heartfelt thanks to my supervisor Dr Suresh Tikoo for giving me the opportunity to pursue my graduate degree and work in his laboratory at the Vaccine and Infectious Disease Organization-International Vaccine Center (VIDO-Intervac). The assistance, encouragement and patience he has extended to me throughout my program have been enormous. This work wouldn't have been possible without his unconditional guidance and persistent help, to which I owe a huge debt of gratitude. I also would like to thank my advisory committee members Dr. Joyce Wilson, Dr Alexander Zakharchouk and Dr Keith Bonham and the graduate chairs Dr Janet Hill and Dr. Vikram Misra for their continuous unreserved guidance and input for the fruition of this work.

I also would like to acknowledge the past and present members of Dr Tikoo's laboratory, especially Satendar Hansra for her technical support. I also owe thanks to all VIDO-Intervac and Department of Veterinary Microbiology staff for their assistance for everything from technical to administrative work. My thanks also go to my past and present laboratory colleagues and fellow graduate students, especially Steve, Sanjeev, Niraj, Amit, Pankaj, Islam, Xin, Kyle, Tekele, Taseen, Andrea, Sughandika, Kuan, Hong, Leo, Sumudu, Musa, Theresa and others for their friendship and enjoyable scientific and non-scientific discussions. I also would like to thank Donna, Rose and Peter for their friendship and cheerful demeanor.

I also would like to thank my parents Tiruye and Eshetu, my sisters Yesem and Lemlem, my borthers Haile and Yohannes, my aunt Ethiopie and my friends Daniel and Fisseha for their unconditional love and encouragement over the years.

Last but not least, I am forever grateful to Prof Shiban Krishan Khar for paving the way to my future.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS USED IN THIS WORK

4E-BPs- eIF4E binding proteins

ARM-Armadillo

BAdV-Bovine adenovirus

BoMtch1-Bovine mitochondrial carrier homolog

BoPSAP- Bovine presenilin-1-associated protein

CaM- Calmodulin

CaMV- Cauliflower mosaic virus

CARD- Caspase recruitment domains

CBP- CREB-binding protein

cNLS- Classical nuclear localization signal

DBP-DNA binding protein

DDX-DEAD box protein

DEAD -Aspargine-glutamine-alanine-aspargine

DMEM- Dulbecco's Modified Eagle's medium

DYNLL- Dynein light chain

E-Early

eIF- Eukaryotic initiation factor

EJC- Exon junction complex

EMCV- Encephalomyocarditits virus

ERα- Estrogen receptor-α

FMD- Foot and mouth disease

FRAP- FKB12-rapamycin associated protein

GST- Glutathione S-transferase

GTase- Guanylyltransferase

HAdV- Human adenovirus

HCV-Hepatitis C virus

HIV- Human immunodeficiency virus

hnRNP A1-Heterogeneous nuclear ribonucleoprotein A1

IBB-Importin β-binding domain

IKK-I-kappa-B-kinase

IKKϵ- IkB kinase-ϵ

Imp-Importin

INF- Interferon

IPS1- IFN-β promoter stimulator-1

IRES-Internal ribosome entry site

ITR-Inverted terminal repeats

L-Late

MAP-Mitogen activated protein

MAVS-Mitochondrial anti-viral signaling

Mda- Melanoma differentiation-associated gene

MDBK- Madin Darby Bovine Kidney

MEM-Minimum essential medium

MLP-Major late promoter

MLTU-Major late transcriptional unit

MMP- Mitochondrial membrane potential

MOI- Multiplicity of infection

mRNP- Mature ribonucleoprotein

mTOR- Mammalian target of rapamycin

N7-MTase- Guanine N-7-methyltransferase

NES-Nuclear export signal

NFBP- NFkB-binding protein

NF-Nuclear factor

NLS-Nuclear localization signal

NPC-Nuvlear pore complex

Nups-Nucleoporins

ORF-Open reading frame

Ori-Origin

PABP-Poly A bindning protein

PC- Preinitiation complex

Pol-Polymerase

PP2A- Cellular phosphatase 2A

PRR-Pattern recognition receptor

RanBP- Ran binding protein

Ras- Ras related nuclear protein

RdRp- RNA-dependent RNA polymerase

RIG-Retinoic acid inducible gene

RNP- Ribonucleoprotein

RRE-Rev response element

rRNA- Ribosomal RNA

RTBV- Rice tungro bacilliform virus

RTPase- RNA 5’ phosphatase

SAM- *S*-Adenosyl-L-methionine

SARS-CoV- Severe acute respiratory syndrome corona virus

SF-2-Superfamily-2

TAP-Tip associated protein

TBK-TANK binding kinase

TLR- Toll like receptor

TP-Terminal protein

TRIF- TIR domain containing adaptor inducing interferon-β

USF-Upstream stimulatory factor

UTR-Untranslated region

VSV-Vesicular stomatitis virus

WNV-West Nile virus

1. LITERATURE REVIEW

The literature review is composed of four main parts; the first part concisely discusses about adenoviruses in general and bovine adenovirus (BAdV)-3 in particular, the second part contains a review on the pathways involved in nuclear import of proteins in eukaryotes and mechanisms used by viruses to import their proteins into the nucleus, followed by the third part which discusses about mRNA translation in eukaryotes and strategies employed by viruses to translate their mRNAs. Finally, the fourth part reviews about the cellular protein DDX3, its role in RNA homeostasis and during virus infection.

1.1.ADENOVIRUSES

Members of the family Adenoviridae are non-enveloped, icosahedral viruses that replicate in the nucleus (Davison et al., 2003; Berk, 2007). Their linear, double stranded DNA genomes are 26-45 kb in size and are characterized by an inverted terminal repeat (ITR) ranging in size from 36 to over 200 bp, and the presence of a terminal protein (pTP) linked to the 5'-ends of the genome (Davison et al., 2003). The ITR (Grable and Hearing, 1992) and pTP play key roles in DNA replication. Adenoviruses were first identified in the early 1950s from the adenoids of humans with acute respiratory infection (Rowe et al., 1953; Hilleman and Werner, 1954). So far, more than 120 serotypes have been isolated from mammals, birds, reptiles and fish (Berk, 2007; International Committee on Taxonomy of Viruses and King, 2012). Most adenoviruses are species specific except those isolated from humans (Roy et al., 2009). Adenoviruses have been used as models for studying virus-cell interactions and helped in the understanding of DNA replication, transcription, splicing and translation processes in eukaryotic cells (White, 1996). Recently adenoviruses have also been studied widely and shown a tremendous potential as viral

vectors because of their ability to infect both dividing and non dividing cells, capacity to package large foreign genes, and relative ease to produce high titer recombinants in cell culture (Shenk, 2001; Russel, 2000). Adenoviral vectors fulfill important criteria of an ideal vaccine vector in terms of efficacy, safety and stability (Tastis and Ertl, 2004) and are the most widely used viral vectors in clinical trials (Arnberg, 2012; Wong et al., 2013).

1.1.1. Transcription and DNA replication in human adenoviruses

Early and late transcription of adenovirus genome is a two phase event occurring before and after DNA replication, respectively. Transcription in adenoviruses is usually followed by a series of splicing events (Russell, 2000). The first region of the viral genome to be transcribed, which appears within 1hr of virus infection, is E1A (Russel, 2009). E1A is a potent trans-activator capable of regulating the transcription of cellular genes and the expression of other early viral proteins in infected cells (Russell, 2000). Though it does not bind DNA directly, it interacts with the DNA binding domains of transcription factors bound to the promoters (Liu and Green, 1994). E1A gene products are also essential for inducing the host cell to enter into S-phase for viral genome replication (Berk, 2007; Turnell and Mymryk, 2006; Ferrari et al., 2008). In addition, E1A can also induce transcriptional repression (Berk, 2005), apoptosis (Frisch and Mymryk, 2002) and suppression of the production of nitric oxide, an antiviral effector of innate immunity by the modulation of the inducible nitric oxide synthase gene increasing the survivability of the virus in the cell (Higashimoto et al., 2006). The pro-apoptotic ability of E1A is counteracted by the E1B proteins, and together E1A and E1B proteins are sufficient to transform cells. E1B 55k protein inhibits apoptosis by repressing the transcription of p53 activated genes (White, 2001).

The E2 region encodes proteins that are essential for viral DNA replication (Berk, 2007). The viral DNA binding protein (DBP) is encoded by the E2A region, while the E2B region encodes the viral DNA polymerase (Pol) and the pre-terminal proteins (pTP) that are indispensable for viral genome replication (Berk, 2007).

The E3 region encodes non-essential genes and transcribes nine messages that are generated by alternate splicing. Its transcription is under the control of the E1A responsive promoter. The proteins encoded in the E3 region are mainly involved in blocking the host's immune responses to viral infection (Horwitz, 2004).

The E4 region produces 18 messages that are produced by alternate splicing and encodes seven proteins that have various functions in the virus infected cells (Weitzman and Ornelles, 2005). These proteins function in regulating viral DNA synthesis, viral RNA splicing, late viral protein synthesis, cell transformation and oncogenecity, early to late switch in viral infection and antagonizing the host immune responses (O'shea et al., 2005; Tauber and Dobner, 2001). E4orf4 binds to the cellular phosphatase 2A (PP2A) and activates the dephosphorylation of SR proteins and regulates adenovirus alternate splicing (O'shea et al., 2005).

The combined action of three viral proteins namely, Pol, pTP and DBP, encoded by the E2 region are essential for adenoviral DNA replication. In addition, two cellular transcription factors namely nuclear factor I (NFI) and NFIII (Oct-1), are required for recruiting the Pol and pTP proteins to its binding site on the viral DNA and stabilizing the protein-DNA interaction (Van Breukelen et al., 2003). The cis-acting DNA sequence that acts as an origin (Ori) of DNA replication is contained in the inverted terminal repeat (ITR) where the preinitiation complex (PC) composed of both viral and cellular proteins are assembled (Coenjaerts et al., 1994). DNA replication normally starts 5 to 8hrs post infection when cells are infected with either human

adenovirus (HAdV)-5 and HAdV-2 at a multiplicity of infection (MOI) of 10 (Berk, 2007). A protein priming at either ends of the linear viral genome initiates DNA replication, which is catalyzed by the viral polymerase that covalently links the β-OH group of a serine residue in pTP and the α-phophoryl group of the terminal dCMP residue (de Jong et al., 2003). The viral Pol then uses the 3'-OH group of the pTP/dCMP complex as a primer for the synthesis of the nascent strand (de Jong et al., 2003). Then, DBP together with the viral Pol elongate the chain by a strand displacement mechanism. The displaced strand can duplicate by base pairing of the ITRs forming a panhandle structure on which a second round of DNA synthesis may be initiated (Wang and Pearson, 1985)

The expression of intermediate proteins IVa2 and pIX, is dependent on DNA replication. However, their transcripts appear earlier than late gene transcripts (Reddy et al., 2010; Ostapchuk et al., 2011) and function as a transcriptional activator of the major late promoter (MLP), which controls the expression the major late transcription unit (MLTU) (Russel, 2009). The primary MLTU in HAdV-5 is differentially spliced into more than 20 different mRNAs that encode viral structural (hexon, penton, fiber, IIIa, V, VI, VII, VIII, IX, and μ), non-structural proteins (52K, 100K, 33K, and 22K) and the core viral protease (Russel, 2009). The late mRNAs are grouped into five families (L1 to L5) in human adenoviruses based on their usage of common polyadenylation sites. In addition, mRNAs transcribed from the MLP contain an identical 5' non-coding segment, which is called the tripartite leader (TPL) that enhances the translation of late viral mRNAs (Berk, 2007).

1.1.2. Bovine adenovirus

Bovine adenovirus (BAdV) was first isolated in the 1960s from an apparently healthy cow (Darbyshire et al., 1965). To date, eleven serotypes (Lehmkuhl and Hobbs, 2008) of BAdV have been isolated from both healthy animals (Darbyshire et al., 1965) and animals with respiratory or enteric diseases (Lehmkuhl et al., 1975; Smyth et al., 1996). The BAdV serotypes are grouped under *Mastadenovirus* (BAdV-1, BAdV-2, BAdV-3, BAdV-9 and BAdV-10) or *Atadenovirus* (BAdV-4, BAdV-5, BAdV-6, BAdV-7, BAdV-8 and strain Rus) genus based on their phylogenetic sequence proximity (Harrach, 2000; Lehmkuhl and Hobbs, 2008). The *Mastadenoviruses* are more closely related to ovine adenoviruses than with each other. Instead, Atadenoviruses are more closely related to each other. The BAdV type 1, 2, 3 grow relatively well in established bovine cell lines. These viruses induce irregularly shaped single nuclear inclusion bodies. The other BAdV types (type 4, 5, 6, 7, 8, and 10) form multiple inclusion of circular shape and can be propagated in low passage cultures of calf testicular or thyroid cells (Bartha, 1969). Darbyshire et al., 1966 also demonstrated that BAdVs can induce tumors in rodents and can transform non-permissive cells in tissue culture.

1.1.3. Bovine adenovirus type-3

Among known BAdV serotypes, bovine adenovirus-3, which belongs to the *Mastadenovirus* genus, is the best characterized (Reddy et al., 1998; Reddy et al., 1999a). Experimental infections of cattle with BAdV-3 produce either subclinical infections with the induction of the production of neutralizing antibodies (Mittal, 1999) or mild respiratory disease (Lehmkuhl et al., 1975). As BAdV-3 lacks virulence, it is being developed and evaluated as a

vector for vaccination and gene therapy for humans and animals (Mittal et al., 1995; Zakhartchouk et al., 1998; Zakhartchouk et al., 1999; Reddy et al., 1998).

1.1.4. Molecular biology of BAdV-3

1.1.4.1. Structure and genome organization

BAdV-3 is a 75nm in diameter, non enveloped icosahedral particle containing a double stranded DNA genome. The genome of BAdV-3 is 34, 446 base pairs with 54% GC content (Reddy et al., 1998). The genome is flanked by 195bp inverted terminal repeats (ITRs) on either end (Shinagawa et al., 1987). Unlike other adenoviruses, the ITRs of BAdV-3 are longer and contain a high GC content (Reddy et al., 1998). The genome of BAdV-3 is organized into early, intermediate and late genes and it has thirty three predicted open reading frames (Reddy et al., 1998; Reddy et al., 1999a; Kulshreshtha, 2009) (Figure 1.1.1). Like other adenoviruses, the packaging domain is located in the left end of the viral genome overlapping the transcriptional control region of E1A (Xing et al., 2003; Xing and Tikoo, 2007). Unlike HAdVs, the E1A region of BAdV-3 genome appears to be required for DNA packaging (Xing et al., 2003; Xing and Tikoo, 2006; Xing and Tikoo, 2007).

The early (E) region is composed of four transcriptional units (E1 to E4). On the other hand, the late region is composed of seven transcriptional units, L1 to L7 (Figure 1.1.1) (Reddy et al., 1998; Reddy et al., 1999a; Kulshreshtha, 2009).

The E1 region of BAdV-3 has two open reading frames encoding for E1A and E1B. Unlike HAdV, E1A and E1B regions are expressed from the same transcriptional unit and have the same polyadenylation site (Reddy et al., 1999). Though the TATA or CAAT boxes are absent in the regions between the left ITR and upstream of the E1A start codon, the expression of

the E1A open reading frame is driven by a promoter located within the ITR (Xing and Tikoo, 2006). The E1A region produces six transcripts by alternate splicing that encode for three proteins namely 211R, 115R and 100R (Reddy et al., 1999a). The E1A proteins are essential for virus replication and functions in trans-activation of the expression of early viral genes (Reddy et al., 1999b; Zhou et al., 2001). In contrast, E1B codes for only two overlapping mRNAs $E1B^{small}$ and $E1B^{large}$ that are homologues of E1B 19K and E1B 55K proteins of HAdV-5, respectively (Zheng et al., 1994). Both $E1B^{small}$ and $E1B^{large}$ proteins appear to be essential for virus replication (Zhou et al., 2001; Zakhartchouk et al., 2001).

The E2 region of BAdV-3, which is transcribed from the complementary strand, codes for proteins that are essential for viral DNA replication (Reddy et al., 1998). The E2 region codes for ORFs E2A and E2B. The E2A transcript codes for the DBP (432 amino acids long), which has 38 to 47% sequence identity with the DBP of other *Mastadenoviruses* (Reddy et al., 1998). The DBP is involved in DNA binding, initiation and maintenance of DNA replication and is expressed in great quantity during the early and late phase of the life cycle of BAdV-3 (Linne and Philipson, 1980, Zhou et al., 2001a). DBP is used for determination of BAdV-3 titer based on immunohistochemical detection of its expression in infected cells (Zhou et al., 2001a). The E2B transcripts encoding DNA Pol and pTPs are 3' co-terminal (Reddy et al., 1998). The BAdV-3 DNA Pol and pTP proteins which have 56 to 62% and 58 to 60% homology, respectively with DNA Pol and pTP proteins of other members of the *Mastadenovirus* genus. The proteins are essential for viral DNA replication (Baxi et al., 1998).

Like in HAdVs, the E3 region of BAdV-3 is located in the region between genes encoding pVIII and fiber proteins (Reddy et al., 1998). BAdV-3 E3 is 1.5Kb, smaller than the E3 region of HAdVs sharing the same polyA with pVIII, 33K and 100K (Mittal et al., 1992).

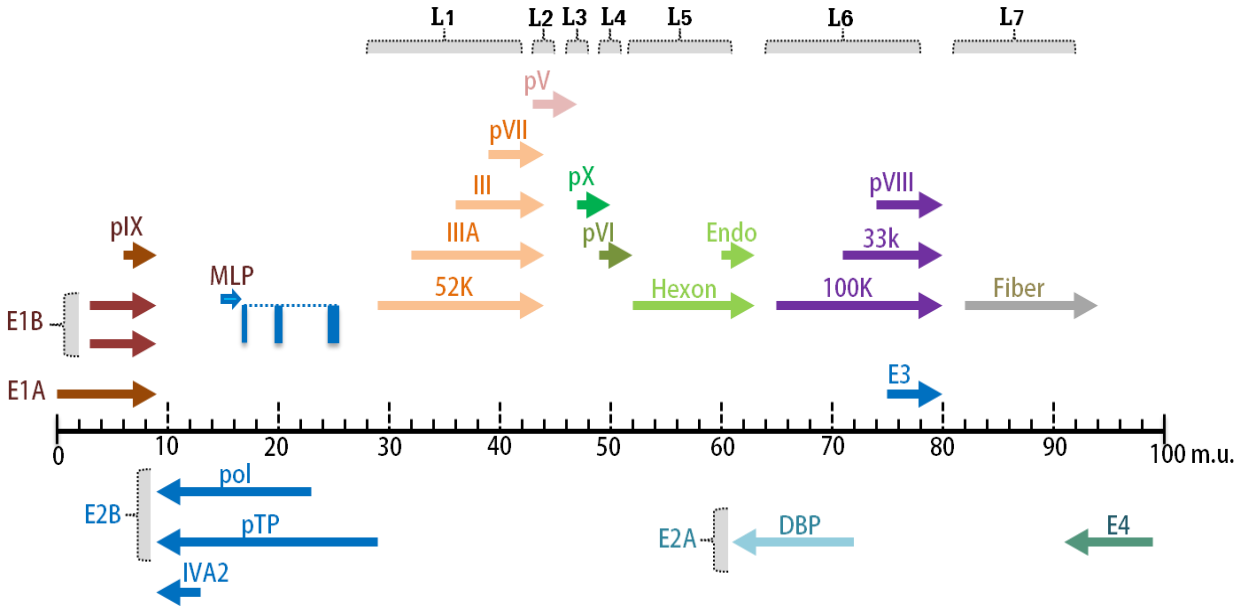


Figure 1.1.1 Transcription map of BAdV-3. Arrows indicate location and direction of transcripts. Major late promoter (MLP); The number depict the map units (m.u); Early (E) transcripts. Late (L) transcripts. (Adapted from Reddy et al., 1998).

Transcription analysis of BAdV-3 E3 detected five transcripts, which have the potential to encode four proteins of 284, 121, 86 and 82 amino acids (Idamakanti et al., 1999). The 121R protein (~14.5kDa) has a limited sequence similarity to the 14.7kDa protein of HAdV-5; however they are serologically and functionally related to each other (Idamakanti et al., 1999; Zakhartchouk et al., 2001). The E3 region is not essential for virus replication (Zakhartchouk et al., 1998; Zakhartchouk et al., 1999) and has been used as a site for insertion and expression of foreign genes (Zakhartchouk et al., 1998; Baxi et al., 2000).

E4 region of BAdV-3 lies in the right end of the genome and is transcribed from right to left on the left strand of the genome (Reddy et al., 1998). Seven transcripts are produced from the E4 region that encodes five proteins namely 143R, 69R, 286R, 143R2 and219R (Baxi et al., 1999). The proteins encoded by the E4 region are not essential for viral replication (Baxi et al., 2001).

Like in HAdVs, the genes encoding for BAdV-3 pIX and IVa2 proteins are classified as intermediate region genes (Reddy et al., 1998). The pIX and IVa2 proteins of BAdV-3 show similarity of 16 to 28% and 29 to 69% with pIX and IVa2 proteins of other adenoviruses, respectively (Reddy et al., 1998). The transcript of BAdV-3 encoding pIX is 3' co-terminal with E1A and E1B regions (Zheng et al., 1999). The IVa2 uses a polyadeynylation signal unique from that of pTP and DNA Pol, while they are 3' co-terminal in HAdVs (Baxi et al., 1998; Zheng et al., 1999). Protein pIX is part of the viral capsid with its C-terminal region exposed on the surface of the capsid, which can be used as a site for incorporation of targeting ligands (Zakhartchouk et al., 2004).

BAdV-3 late regions encode both structural and non structural proteins. Like in HAdVs, the late regions of BAdV-3 are transcribed under the control of the MLP that contains the

canonical TATA box, inverted CAAT box, the transcriptional factor USF binding site and the initiator element (Reddy et al., 1998). Like HAdVs, all the late transcripts of BAdV-3 contain the non-coding TPL (205 nucleotides in length) sequence at their 5' ends (Reddy et al., 1998). The BAdV-3 TPL is a spliced product of three small exons and is located within the non coding strands of the DNA Pol and the pTP genes, respectively (Reddy et al., 1998). The first exon is 40 nucleotides long and is located adjacent to the MLP, the second is 78 and the third is 87 nucleotides long. The late transcripts of BAdV-3 are divided into seven families (L1 to L7) based on usage of common 3' polyadenylation sites (Reddy et al., 1998).

The L1 region comprises four late region genes that have common poly(A) signals (52K, IIIA, III, and pVII). The 52K protein is expressed as 40kDa nuclear protein in virus infected cells. The 52K protein uses the classical importin α/β pathway to localize into the nucleus by preferentially binding with importin-α3 and also requires nuclear localization signal located at amino acids 102 to 110 (Paterson et al., 2012). BAdV-3 52K interacts with the viral pVII protein and the cellular NFkB-binding protein (NFBP) (Paterson, 2010). BAdV-3 IIIa protein shares sequence identities of 25 to 57% to the IIIa proteins of other adenoviruses. Protein IIIa has a predicted protease cleavage site located 19 residues from the C-terminus (Reddy et al., 1998). The BAdV-3 pIII is a penton base protein and contains MDV (Methionine-Aspartic acid-Valine) motif instead of the RGD (Arginine-Glycine-Aspartic acid) motif found in HAdV-5 (Reddy et al., 1998). pVII of BAdV-3 is highly basic like other proteins having DNA binding function and has a consensus protease cleavage site located 19 nucleotides from the N-terminus of the protein (Reddy et al., 1998). BAdV-3 pVII contains a functional mitochondrial localization signal, which helps pVII to locate to Mitochondria (Anand et al., 2014). Mitochondrial localization of BAdV-3 pVII appears to help in the retention of Ca2+ in the mitochondria thus increasing the levels of

ATP and maintaining the mitochondrial membrane potential (MMP) in transfected and virus infected cells (Anand et al., 2014).

The L2 region of BAdV-3 codes for a single transcript that expresses pV protein. It contains a bipartite nuclear localization signal in the central region of the protein. It shares a protein sequence similarity of 28 to 41% with other members of the *Mastadenovirus* genus. In addition, the pV sequence has a high content of basic amino acids (Reddy et al., 1998)

The L3 region of BAdV-3 encodes a single transcript that expresses pX (μ) protein. The protein contains two predicted adenovirus specific protease cleavage sites. pX is rich in basic amino acids and contains a conserved nuclear localization signal (Reddy et al., 1998).

The L4 region produces a transcript that codes for protein pVI. It shares 32 to 39% protein sequence similarity with the pVI proteins of other adenoviruses. It is a minor component of the virion and contains two consensus viral cleavage sites after residues 33 and 252 (Reddy et al., 1998).

The L5 region of BAdV-3 encodes hexon and the viral protease proteins. In contrast to other adenoviruses, hexon and proteases ORFs do not overlap. BAdV-3 hexon has protein sequence identity of 66 to 71% with other adenoviruses (Reddy et al., 1998). BAdV-3 hexon is expressed as a 98kDa protein in infected cells (Kulshrestha et al., 2004). BAdV-3 protease shares a 60% sequence identity with proteases of other adenoviruses (Reddy et al., 1998). DNA binding residues of BAdV-3 protease and HAdV-2 protease are conserved (Gupta et al., 2004)

The L6 region of BAdV-3 encodes the structural protein pVIII and the non-structural proteins 100K, 22K and 33K (Reddy et al., 1998; Kulshreshtha, 2009). Protein 100K is the largest non-structural protein encoded by members of *Mastadenoviruses*. BAdV-3 100K shares 27 to 52% protein sequence identity with other adenoviruses (Reddy et al., 1998; Kulshreshtha et

al., 2004). 100K protein appears to localize both in the nucleus and cytoplasm with the nuclear localization signal located between aminoacids 624 to 637. BAdV-3 protease cleaves 100K protein at two identified adenovirus specific protease cleavage sites; amino acids 740-745 and 781-786 and only the C-terminal cleaved portion of 100K localizes in the nucleus. However, cleavage is not essential for virus replication, but was shown to be involved in lysis of infected cells for release of mature virions. 100K protein also interacts with BAdV-3 33K protein and cellular dynein light chain (DYNLL)-1 (Makadiya, 2013).

33K protein is a product of spliced transcript and it shares N-terminus 138 amino acids with 22K protein. Both 22K and 33K proteins localize in the nucleus and play a role in virus assembly, specifically in efficient capsid-viral DNA interactions (Kulshreshtha et al., 2004). The 33K protein of BAdV-3 trans-activates the MLP (Kulshreshtha, 2009). The BAdV-3 33K interacts with bovine presenilin-1-associated protein / mitochondrial carrier homolog 1 (BoPSAP / BoMtch1) and the BAdV-3 proteins 100K and pV proteins (Kulshreshtha, 2009). Deletion mutant analysis of 33K protein indicated that amino acids 201 to 240 containing RS (Arginine/Serine) repeats are required for nuclear localization and its nuclear import is mediated by both transportin and importin α/β dependent pathways. Moreover, mutations of RS from wild type BAdV-3 proved to be lethal (Kulshreshtha et al., 2014). Association of BAdV-3 33K protein with BoPSAP appears to inhibit the pro-apoptotic activity of BoPSAP suggesting a role of BAdV-3 33K as an anti apoptotic protein (Kulshreshtha, 2009). pVIII of BAdV-3 is a minor capsid protein with 216 aminoacids and it contains two predicted viral protease cleavage sites (Reddy et al., 1998).

The L7 region of BAdV-3 encodes fiber protein which show low protein sequence identity (22-27%) with fiber protein encoded by other adenoviruses. The fiber protein

determines the tissue tropism of BAdV-3 (Wu and Tikoo, 2004). The BAdV-3 fiber is expressed as a102kDa nuclear protein, which is comprised of tail, shaft and knob regions. The shaft region of BAdV-3 fiber protein contains a very long shaft region as compared to HAdV-5 and has several bends (Ruigrok et al., 1994). The protein contains a nuclear localization signal in the N-terminal region, which appears essential for efficient replication of BAdV-3 (Wu et al., 2007).

A brief summary of the early, intermediate and late gene expression of BAdV-3 including a short description of the known functions and properties of each viral protein is summarized in Tables 1.1.4.1, 1.1.4.2 and 1.1.4.3.

1.2.NUCLEAR IMPORT OF PROTEINS IN EUKARYOTES

The different compartments of the eukaryotic cell are separated by membranes. The double membrane nuclear envelope separates the nucleus and the cytoplasm; however, molecules should be selectively transported between the two compartments for the cell to function properly. For example, after transcription occurs in the nucleus, the RNA should be exported into the cytoplasm for protein translation to take place. Similarly, proteins that have functions in the nucleus needs to be imported into the nucleus after being synthesized in the cytoplasm (Cook et al., 2007).

1.2.1. Nuclear pore complex

The only gate of molecules between the cytoplasm and the nucleus is a massive proteinacious channel termed the nuclear pore complex (NPC) that is rooted in the nuclear membrane (Cook et al., 2007; Vasu and Forbes, 2001). The NPC is a huge multi-protein complex with a total mass of

Table 1.1.4.1. Early BAdV-3 gene expression

Region	ORF	Proteins	Remarks
E1	E1A	211aa	The proteins don't have predicted classical nuclear localization signal. Localize into the nucleus through their interaction with cellular proteins. They are required for virus replication and trans-activation of early viral genes (Reddy et al., 1999a; Zhou et al., 2001)
		115aa	
		100aa	
	E1B	E1Bsmall	E1B has independent promoter and polyadenylation site from E1A. E1B small is a homologue of HAdV-5 19kDa protein. Localizes in the nucleus and is essential for virus replication, but dispensable in some cells types (Zheng et al., 1994: Zhou et al., 2001)
		E1Blarge	Homologue of HAdV-5 55kDa protein. Localizes in the nucleus and is required for virus replication (Zakhartchouk et al., 2004)
E2	E2A	DNA binding protein (DBP)	Has a conserved "C" terminus which is involved in DNA binding, initiation and maintenance of DNA replication and transcriptional control of the major late promoter (Linne and Philipson, 1980; Zhou et al., 2001a)
	E2B	Viral DNA polymerase	Localizes in the nucleus by forming a heterodimer with pTP. Has a conserved zinc finger motifs as is in other *Mastadenovirus* genus that are essential for viral DNA replication (Baxi et al., 1998)
		Pre-terminal protein (pTP)	Has a strong nuclear localization signal. It contains a conserved YSRLVYR motif that is required for protein priming of DNA replication initiation (Baxi et al., 1998)
E3		Four proteins of 284, 121, 86 or 82 amino acids	Not essential for virus replication. Replication competent E3 deleted BAdV-3 has been generated. The 284R protein is glycosylated. The E3 gene products of HAdV-5 suppress host immune responses (Weeks and Jones, 1985; Wold et al., 1995; Horwitz, 2004). 121R protein was shown to protect mouse cells from TNF-mediated cell lyses (Zakhartchouk et al., 2001)
E4	ORF1	143R	Not required for BAdV-3 replication on cultured cells. 143R, 69R and 143R2 do not have homologues in other adenoviruses and appears to be unique to BAdV-3 (Baxi et al., 2001)
	ORF2	69R	
	ORF3	286R	
	ORF4	143R2	
	ORF5	219R	

Table 1.1.4.2. Intermediate BAdV-3gene expression

Region	ORF	Proteins	Remarks
E1	E1B	pIX	Component of viral capsid. The C-terminus is exposed on the surface of the viral capsid and it acts as a site for incorporation of ligands (Reddy et al., 1999b; Zakhartchouk et al., 2004)
E2	E2B	IVa2	Has a polyadenylation signal unique from that of pTP and DNA Pol (Baxi et al., 1998; Zheng et al, 1999)

Table 1.1.4.3. Late BAdV-3 gene expression

Region	Proteins	Remarks
L1	52K	Predominantly localizes into the nucleus through the classical importin α/β pathway. 52K interacts with the viral pVII protein and the cellular NFkB-binding protein (NFBP) (Paterson et al., 2012)
	IIIa	Contains a putative viral protease cleavage site.
	Penton	Lacks the conserved RGD motif for integrin binding. It also lacks a leucine-aspartic acid-valine motif but contains a methionine-aspartic acid-valine (MDV) motif (Reddy et al., 1998)
	pVII	pVII is the major coat protein and has a cleavage site for the adenovirus protease (MYGG ^A) (Reddy et al.,1998)
L2	pV	pV is a core protein and it contains a bipartite nuclear localization signal (Reddy et al., 1998)
L3	pX (μ)	Contains two viral protease specific cleavage sites at residues 31 and 50 and is rich in basic amino acids and has a conserved nuclear localization signal (Reddy et al., 1998)
L4	pVI	pVI is a minor component of the virion. Contains two consensus viral protease cleavage sites after residues 33 and 252 (Reddy et al., 1998)
L5	Hexon	Unlike in other adenoviruses, BAdV-3 hexon has no overlapping ORFs. Like other adenoviruses it has loops exposed on the external surfaces that contains hyper variable regions(Hu et al., 1984; Crawford-Miksza and Schnurr, 1996; Reddy et al., 1998)
	Viral protease	Shows homology with proteases of other adenoviruses (Reddy et al., 1998). DNA binding residues of the protein are conserved between BAdV-3 and HAdV-2 (Gupta et al., 2004)
	100K	The largest non structural protein. Cleaved at two sites by

L6		adenovirus protease. The C-terminal fragment localizes in the nucleus. 100K interacts with BAdV-3 33K and the cellular protein DYNLS-1 (Makadiya, 2013).
	22K	Non structural protein that localizes in the nucleus and involves in capsid assembly, especially in the encapsidation of viral DNA (Kulshreshtha et al., 2004).
	33K	33K is also a non structural protein that localizes in the nucleus. It helps in capsid assembly and efficient DNA capsid interaction. In addition it is involved in *trans* activation of the MLP (Reddy et al., 1998; Kulshreshtha, 2009).
	pVIII	pVIII is a structural protein that contains two viral protease cleavage sites (Reddy et al., 1998)
L7	Fiber	The fiber has a nuclear localization signal. 22-27% homologous with fiber of other adenoviruses. It has a tail, shaft and knob domain. The shaft is quite long with several bends (Ruigrok et al., 1994; Reddy et al., 1998).

~125MDa (Adams and Wente, 2013) that lies across the nuclear membrane (Wente and Rout, 2010) and harbors ~65nm long central aqueous channel. It is mainly composed of glycoprotein called gp210 (Kahms et al., 2011). The NPC has an octagonal symmetry and can be divided into three basic components; the nuclear basket, the central core and the cytoplasmic fibrils (Figure 1.2.1) (Beck et al., 2004). The central framework forms a cylindrical structure with eight cytoplasmic filaments and the nuclear basket is made up of eight filaments that join into a distal ring. The NPC is asymmetric with respect to its cytoplasmic and nuclear extensions (Kahms et al., 2011). Each NPC is composed of ~30 proteins called nucleoporins (Nups) that line the central transport channel of the pore. They are present in copies of eight or multiples of eight (Rout et al., 2000). About two thirds of the Nups appear to be evolutionarily conserved between yeast and mammals (Vasu and Forbes, 2001; Allen et al., 2000). The Nups contain FG, FxFG and GLFG repeats that provide binding sites for nuclear import and export factors, and are directly involved in energy dependent nuclear transport (Bayliss et al., 2000; Bayliss et al., 2002). The Nups are mainly divided in to three classes namely; transmembrane proteins, beta propeller folds, and alpha solenoid folds (Tran and Wente, 2006; Lim et al., 2008)

1.2.2. Nuclear import pathways

Nuclear protein import is a directional active translocation of proteins from the cytoplasm into the nucleus (Marfori et al., 2011). Proteins that are transported in an energy dependent mechanism need to possess a special sequence called nuclear localization signal (NLS) (Wagstaff and Jans, 2009) and require soluble cytosolic nuclear transport receptors and proteins of the nuclear pore complex (Sorokin et al., 2007).

The prototype monopartite and bipartite cNLS are the NLS of SV-40 T antigen (PKKKRKV) and nucleoplasmin protein (KRPAATKKAGQAKKKK), respectively (Lange et al., 2007). The cNLS makes hydrogen bonds with conserved aspargines of the Imp-α H3 helices and the NLS peptide insert into the cNLS binding groove of Imp-α containing two lysine binding pockets. The lysine side chains of the NLS and the tryptophans of Imp-α involve in hydrophobic interactions with each other. In addition, the basic NLS lysines interact with the surrounding acidic residues of Imp-α (reviewed in Cook et al., 2007). Furthermore, cargos that carry non classical NLS can also directly interact with Imp-β and are imported into the nucleus in Imp-α independent/Ran dependent mechanism. Examples include, NLS of Rev and Tat proteins of HIV (Gu et al., 2011; Truant and Cullen, 1999), the ribosomal proteins L23a and L5 (Jakel and Gorlich, 1998), Cyclin B1 (Moore et al., 1999) and other several proteins like the transcription factors CREB, Jun and Fos (Forwood et al., 2001).

1.2.2.1. Nuclear localization signals

Smaller macromolecules, ions and metabolites passively diffuse into the nucleus. While the import of molecules larger than 40kDa is mediated by special amino acid targeting sequences on import substrates called Nuclear Localization Signals (NLS). The best characterized nuclear localization signals termed “Classical (c)” NLS are composed of one cluster (monopartite) or two clusters (bipartite) of basic amino acids separated by ~10-12 amino acid spacer (Lange et al., 2007). Although variety of proteins uses cNLS for localization to the nucleus, many nuclear proteins use unconventional NLS which do not match with the well defined consensus cNLS (reviewed in Fried and Kutay, 2003). Among the proteins annotated in GenBank, 57% of the proteins that are identified to localize in the nucleus contain cNLS (Lange et al., 2007).

However, recent reports suggest that many proteins utilize a number of non classical NLSs for transport to the nucleus (Christophe et al., 2000). A variety of other sequence motifs have been identified that direct proteins into the nucleus (Gorlich, 1997). For example, the c-myc protein contains non-classical NLS consisting of nine amino acids, PAAKRVKLD, of which only three are basic amino acid residues (Dang and Lee, 1988). The NLS of the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) known as M9 is composed of 38 amino acids, which is essential for both nuclear import and export (Siomi and Dreyfuss, 1995). The NLS of the ribosomal protein rpL23a called BIB, which consists of a basic residues rich 42 amino acid sequence (Jakel and Gorlich, 1988).

1.2.2.2. Protein transport receptors (importins)

Selective transport of macromolecules from the cytoplasm to the nucleus is mediated by soluble cytosolic nuclear import factors called karyopherins or importins. These proteins have also been referred to as NLS receptors or Nuclear Pore Targeting Complex (Gorlich and Mattaj, 1996). The NLS of cargo proteins is recognized and bound by the 60kDa protein named importin-α in the cytoplasm that has six different isoforms in mammals, displaying differing tissue expression patterns, and act as an adaptor protein for importin-β mediated nuclear transport (Pemberton and Paschal, 2005). Importin α- interacts directly with the 90kDa importin-β (which is also termed as p97, karyopherin-β, and PTAc in mammals [Sorokin et al., 2007]) through its importin-β-binding domain (IBB) which is located in the amino terminus of importin-α between residues 10 and 55 (Gorlich et al., 1996a; Xu et al., 2010). In addition to IBB, importin-α also contains a highly structured domain consisting of ten tandem Armadillo (ARM) repeats in its C-terminus. The ARM repeats are relatively hydrophobic and contain three

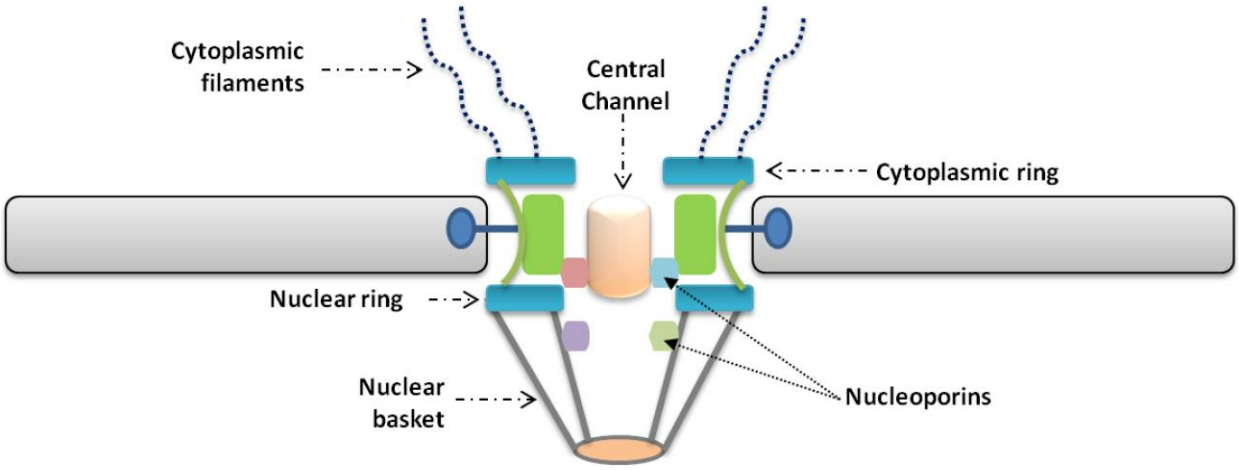


Figure 1.2.1 Schematic diagram of the nuclear pore complex (Adapted from Allen et al., 2000)

α-helices (termed H1, H2, and H3). The ARM repeats assemble into a spherical, twisted structure with a long groove that serves as a binding site for monopartite and bipartite NLS.

These structures show a higher degree of conservation between different variants of importin-α (Marfori et al., 2011). The tenth ARM repeat also serves as a binding site for the exportin CAS (cellular apoptosis susceptibility) (Herold et al., 1998). The IBB and ARM repeat domains are conserved across different variants of importin-α. However, other regions tend to be poorly conserved and have therefore been termed variable regions (Goldfarb et al., 2004). In addition to the sequences that bind to importin-β and NLS, importin-α possesses a leucine-rich nuclear export signal (NES) for its quick return to the cytoplasm (Cortes et al., 1994).

IBB domain interacts either in *cis* with the cNLS binding groove or in *trans* with importin-β. When importin-α is free in the cytoplasm (not bound to an NLS cargo), its IBB is not exposed to importin-β. Through these interactions it acts as a competitive inhibitor to regulate the binding of the NLS cargo to the nascent targeting complex. By doing so, it controls the ternary complex assembly and disassembly (Goldfarb et al., 2004). Importin-β1 is a family of closely related proteins, including nuclear export factors Crm1 and CAS, and the nuclear import factor transportin, but only importin-β1 participate in importin-α dependent nuclear import processes (Chook and Suel, 2011). Importin-β1 interacts with the NLS adaptor importin-α (Cingolani et al., 1999), Ran (Chook and Blobel, 1999; Lee et al., 2005), cargo proteins (Mitrousis et al., 2008) and Nups (Bayliss et al., 2000). Its large surface area and the inherent flexibility of the solenoid structure enable it to bind with a wide variety of proteins (Forwood et al., 2010). importin-β1 contains 19 tandem HEAT repeats (named after the proteins in which they were first identified:Huntingtin, Elongation factor 3, the A subunit of protein phosphatase A, and Tor1 kinase), each consisting of two α-helices in a hairpin structure. They are arranged in

a superhelix comprising two helices; the helix A (convex face) and helix B (concave face). The other binding partners of importin-β1 interact with the concave face of importin-β1 except Nups which bind to the convex face (reviewed in Marfori et al., 2011). The interaction between importin-β and importin-α involves HEAT repeats 7-19 (Cingolani et al., 1999).

The cargo-importin-α/β ternary complex is then docked into the cytoplasmic periphery of the NPC via the action of importin-β (Pemberton and Paschal, 2005). Importin-β binds to GLFG or FXFG repeat domains of different Nups *in-vitro* and mediates the interactions with the NPC that drive translocation into the nucleus (Bednenko et al., 2003). Lastly, the import-ligand complex has to be moved ~100nm across the NPC by an energy dependent, Ran dependent mechanism. Then, nuclear import is completed by the dissociation of the import substrate and the nuclear import factors (Stewart, 2007). The dissociation of the trimeric complex is mediated, in part, by the binding of the small nuclear GTPase Ran-GTP to importin-β. Cargo proteins can also be transported through direct interactions with importin-β (Xu et al., 2010). The schematic of Imp α/β heterodimer pathway mediated nuclear import of proteins is shown in Figure1.2.2.

1.2.2.3. Nuclear import cycle

A small GTPase Ran is essential for the energy dependent import and export of molecules across the NPC (Stewart, 2007). The hydrolysis of GTP by Ran (Ras related nuclear protein) is required for the translocation of the cargo-Importin-α/β complex through the NPC. This process is facilitated by NTF2 (the nuclear transport factor 2). Ran is a 25kDa protein that is predominantly located in the nucleus (Moore, 1998). The unique feature of the RanGTPase cycle is the predominantly cytoplasmic pattern of cellular distribution of its regulators RanGAP (the main GTPase activating protein) and RanBP1 (Ran binding protein), which together can achieve

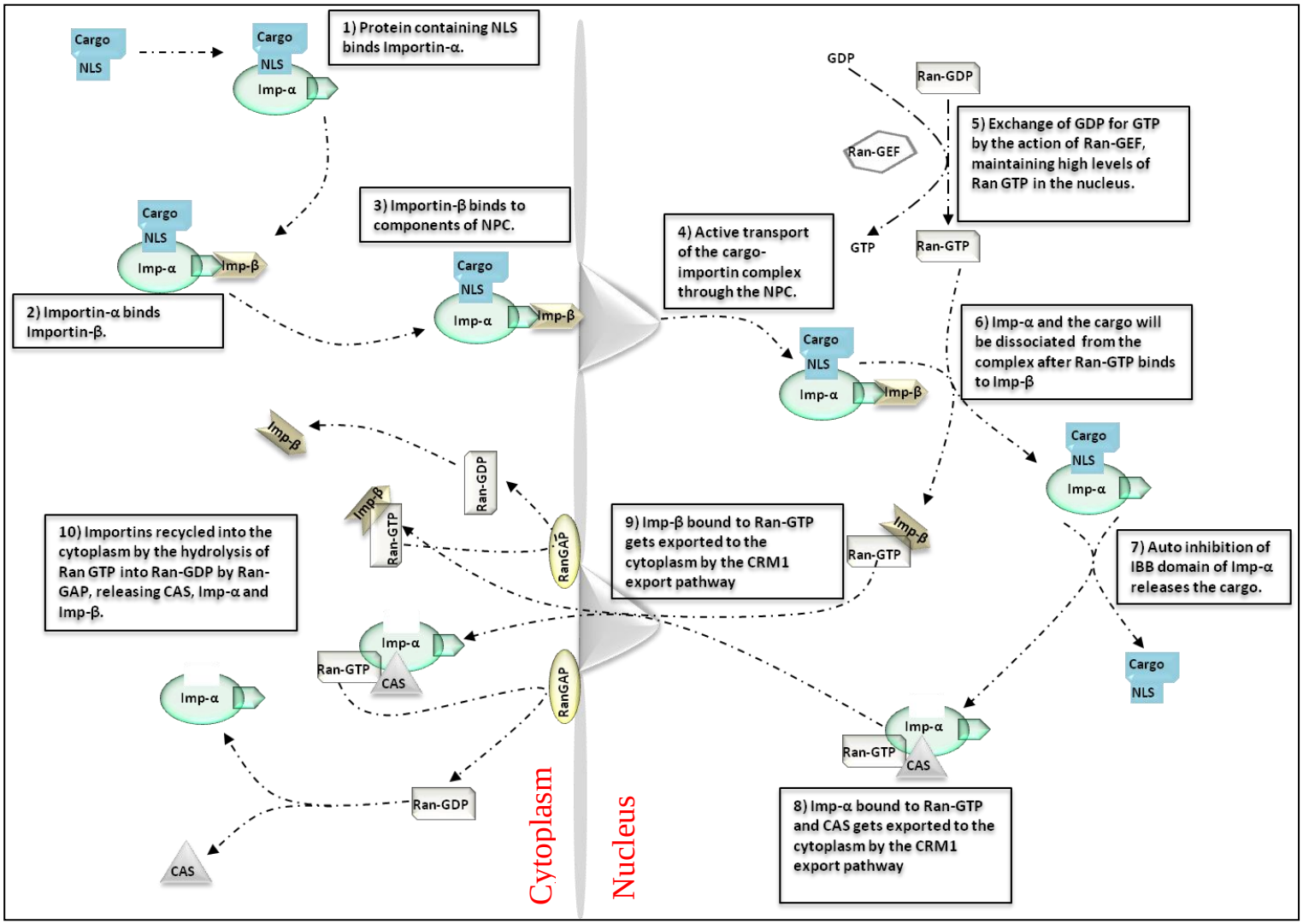


Figure 1.2.2 The mechanism of importin α/β hetetrodimer dependent/Ran dependent import of proteins into the nucleus (Adapted from Stewart, 2007)

maximum GTPase activity. In contrast, RCC1 (the nucleotide exchange factor of Ran) is predominantly nuclear. As a result, there is a steep gradient of RanGTP across the NPC with a high level in the nucleus and a low level in the cytoplasm (Sorokin et al., 2007). This controls the formation of Importin-cargo complexes by facilitating the assembly in the cytoplasm (low Ran-GTP) and by facilitating the dissociation in the nucleus (high Ran-GTP) (Stewart, 2007).

The dissociation of the cargo-Importin-α/β complex starts with the interaction of Ran-GTP to three different regions of importin-β, which results in an allosteric conformational change of Imp-β that finally displaces the cargo and hinders the interaction between IBB domain of Imp-α and Imp-β (Lee et al., 2005; Chook and Blobel, 1999).

Importins are exported back to the cytoplasm as complexes with Ran-GTP by binding with the nuclear export proteins (CAS, exprotin1/Crm1p). Once in the cytoplasm, the RanGAP and the RanBP1 (exclusively cytoplasmic) / RanBP2 located on the cytoplasmic fibrils of the NPC) accelerate the conversion of RanGTP into RanGDP resulting in the dissociation of Ran from the complex which ultimately releases its cargo from the export receptor. Ran is actively imported back to the nucleus by the action of the transport factor p10/NTF-2, which specifically binds to RanGDP and converts Ran-GDP to Ran-GTP by nucleotide exchange releasing Ran from p10/NTF2 (Sorokin et al., 2007).

1.2.2.4.Ran independent nuclear transport of proteins

There are alternate nuclear import pathways that have been described, which are totally independent of Ran. For instance, importin-β1, β-catenin (Wagstaff and Jan, 2009), transportin-1(Nakielny and Dreyfuss, 1998), transportin 3 (Kataoka et al., 1999) can be imported into the nucleus independent of Ran, but by directly binding with proteins of the NPC. The nuclear

export factor Crm1 also binds to Nup214 and migrates into the nucleus without requiring Ran (Fornerod et al., 1997). The nuclear import of calcium binding protein calmodulin (CaM) depends on the concentration of Ca^{2+} in the cytoplasm and CaM binding proteins are translocated through the NPC in the absence of Ran (Hanover et al., 2009). In addition, cNLS containing proteins that are recognized by CaM can be transported into the nucleus without GTP hydrolysis, but in the presence of ATP in CaM dependent pathway (Sweitzer and Hanover, 1996).

1.2.2.5.Pathways for nuclear import of adenoviral proteins

Adenovirus genome replication and capsid assembly take place in the nucleus. Hence, viral proteins synthesized in the cytoplasm need to be imported into the nucleus to achieve replication, the assembly of capsids and ultimately the production of infectious progeny virus. Even though extensive work has not been done characterizing the mechanisms of nuclear import of adenovirus proteins, studies on small number of adenovirus proteins have shown the use of different nucleocytoplasmic shuttling receptor pathways.

Structural proteins: Protein pVII of HAdV-5 has been shown to contain three NLS containing regions (amino acids 82 to 114, 115 to 169 and 155 to 199) (Wodrich et al 2006). Protein pVII and its NLS domains specifically bind to nuclear import receptors (Imp-α, Imp-β, Imp-7 and transportin). Another report also demonstrated that pVII interacts with Imp-α, Imp-β, Imp-7 and transportin, but it is only transportin that interacts with mature VII (Hindley et al., 2007). Since pVII is tightly associated with the adenovirus genome and can act as an adaptor connecting the genome with nuclear import machinery and help in the delivery of adenovirus genome into the host nucleus preferentially through transportin mediated pathway (Lee et al., 2003; Wodrich et al., 2006). Protein V of human adenovirus is translocated into the nucleus in an

energy dependent mechanism by either transportin (1, 2 or 3) or Imp-α/β heterodimer mediated import pathway (Hindley et al., 2007).

The hexon protein that constitutes the largest mass of adenovirus is cytoplasmic when expressed alone and does not have known NLS (Russell and Kemp, 1995). The nuclear import of hexon in virus infected cell is mediated by protein pVI, which acts as an adaptor linking hexon to the transport receptors and shuttles between the cytoplasm and the nucleus (Wodrich et al., 2003). The C-terminal peptide of pVI contains the NLS and NES, which is cleaved during virus maturation and is sufficient to import carrier molecules through the Imp-α/β import pathway (Wodrich et al., 2003). The fiber protein of BAdV-3 contains a NLS that are composed of basic residues (^{14}PYKAKRP^{20}). Substitution of the basic (KKR) residues with acidic (EEE) residues inhibits nuclear import of the protein. Mutating the NLS of the fiber in BAdV-3 did not affect formation of viral particles, but reduced virus growth which may be due to poor formation of infectious viral particles (Wu et al., 2004).

Non-structural proteins*:* Adenovirus E1a protein that functions in transcriptional control of adenovirus genes contains a pentameric NLS in its C- terminus, which directs nuclear accumulation of the protein (Lyons et al., 1987), mediated by Imp-α3 (Kohler et al., 2001). Similarly, the E1B-55K protein contains a C-terminal NLS and an N-terminal leucine-rich NES and shuttles between the nucleus and the cytoplasm (Pollard and Malim, 1998). The N- terminus of HAdV- 2 E4-6/7 protein (Schaley et al., 2005) and the C-terminus of HAdV-5 E4-ORF6 protein (Orlando and Ornelles, 1999) contain an arginine-rich nuclear localization signal.

The nuclear import of BAdV-3 L1-52K protein is mediated by Imp-α3 and three basic residues (^{105}RKR^{107}) are essential for nuclear localization of the protein (Paterson et al., 2012). Likewise, the C- terminus of HAdV-5 L4-100K protein contains a functional NLS (Cuesta et al.,

2004). Reports also indicate that 100K protein helps in the nuclear localization of hexon protein (Hong et al., 2005).

1.3. mRNA TRANSLATION IN EUKARYOTES

Translation of mRNA is a post transcriptional mechanism that controls gene expression. It occurs after mRNA biogenesis, which typically starts with the transcription of mRNAs in the nucleus, followed by mRNA processing that includes capping at the 5' end, splicing and polyadenylation, ribonucleoprotein (RNP) assembly and export of mature RNPs (mRNP) to the cytoplasm via the NPC.

The translation process is generally divided into initiation, elongation and termination stages. However, translation is principally regulated at the initiation stage. Translation initiation is considered a rate limiting factor for overall protein synthesis in eukaryotes (Sonenberg and Hinnebusch, 2009). While the majority of eukaryotic mRNAs are translated by a scanning mechanism, initiation of few mRNAs is mediated by internal ribosome entry sites (IRES) (Jackson et al., 2010).

1.3.1. Cap dependent translation

Translation initiation involves several steps of protein-mRNA and protein-protein interactions. Structural elements like the 5'cap and the 3' polyA tail are important determinants of these interactions (Van Der Kelen et al., 2009). The initiation process starts with the interaction of eukaryotic initiation factor (eIF)2-GTP complex with Met-tRNAi forming eIF2/GTP/Met-tRNAi ternary complex, which is bound by a preformed 40S-eIF3-eIF1A leading to the formation of 43S pre-initiation complex (PC) (Lopez-Lastra et al., 2005). The binding of

the ternary complex with the 40S ribosome is an essential step in translation initiation. The ternary complex then binds with other eIFs and facilitates the delivery of Met-tRNAi into the P site of the 40S ribosome (Hinnebusch, 2000). This is followed by recruitment of 43S pre-initiation complex by eIF3 and eIF4F complex to the 5' end of the mRNA.

The binding of 43S complex to the mRNA is enhanced by the recognition of m^7G cap at the 5'- end of mRNA by the cap binding protein subunit of eIF4F, eIF4E and eIF3 bound to 43S complex. Then, the mRNA bound ribosomal complex scans through the 5' untranslated region (UTR) of the mRNA untill the recognition of first AUG to form a 48S initiation complex through base pairing of the initiation codon to the anticodon of initiator tRNA. Finally, the 60S ribosomal subunit will join to form a functional 80S ribosome, leaving Met-tRNAi in the ribosomal P site (Sokabe et al., 2011) (Figure 1.3.1).

1.3.2. Mechanism of recruitment of 43S preinitiation complex (PC)

The 5' ends of most eukaryotic mRNAs contain a special sequence called m^7G cap, which facilitates mRNA translation initiation by acting as a recruitment site for 43S PC. It is also involved in splicing and nucleocytoplasmic transport of mRNAs and protecting mRNAs from 5' to 3' exonuclease degradation (Jackson et al., 2010). ATPase and the helicase activity are required for mRNAs that contain secondary structures at their 5' UTR to facilitate the recruitment of the 43S PC at the m^7G Cap and for the subsequent scanning (Pestova and Kolupaeva, 2002). The eIF4F complex (which is composed of the cap binding complex eIF4E, the scaffold protein eIF4G, and the ATP dependent RNA helicase eIF4A) is central in mediating the recruitment of 43S PC to the 5' Cap (Jackson et al., 2010) (Figure 1.3.1).

The eIF4F subunit eIF4E directly interacts with the 5' cap structure of mRNAs and recruit the translation machinery to the 5' end of mRNAs. Under normal physiological conditions, eIF4E is bound by 4E-BPs (4E-binding proteins) (Rong et al., 2008). The binding affinity of 4E-BPs for eIF4E is regulated by the phosphorylation of the specific serine/threonine residues in 4E-BP proteins (Fischer, 2009; Fadden et al., 1997). The hypo-phosphorylated 4E-BP strongly binds to eIF4E and drastically reduces translation initiation; however, when hyper-phosphorylated, its binding to eIF4E is blocked and translation initiation is enhanced (Jia et al., 2012). Exposure of the cells to the external stimuli like cytokines, hormones, mitogens, growth factors induce hyper-phosphorylation of 4E-BPs, while deprivation of nutrients or growth factors results in dephosphorylation of 4E-BPs (Holcik and Sonenberg, 2005). The FRAP/mTOR (FKB12-rapamycin associated protein/mammalian target of rapamycin) pathway is required for the phosphorylation 4E-BP1 (Gingras et al., 2001). Generally, while the eIF4F formation is presumed to be largely regulated by eIF4E (Rau et al., 1996), the eIF4E activity is controlled by the regulation of its transcription, phosphorylation of the eIF4E protein (i.e. eIF4E phosphorylation is correlated with increased translation rate) and by its interaction with translational repressor proteins (Pause et al., 1994). The eIF4E is phosphorylated by MAP (mitogen activated protein) kinase signal integrating kinases Mnk1 and Mnk2 at Ser209 in mammals (Raught and Gingras, 1999).

The scaffolding protein eIF4G couples the mRNA and the translation machinery by multiple protein-mRNA and protein-protein interactions. eIF4G contains binding sites for eIF4A, eIF4E, eIF3 and poly A binding protein (PABP) (Jackson et al., 2010). The N-terminus of eIF4G binds with eIF4E (Mader et al., 1995), and the middle and C- terminus interacts with eIF4A (Imataka and Sonenberg, 1997). The binding of eIF4G with eIF3 (part of the 43S PC) brings the

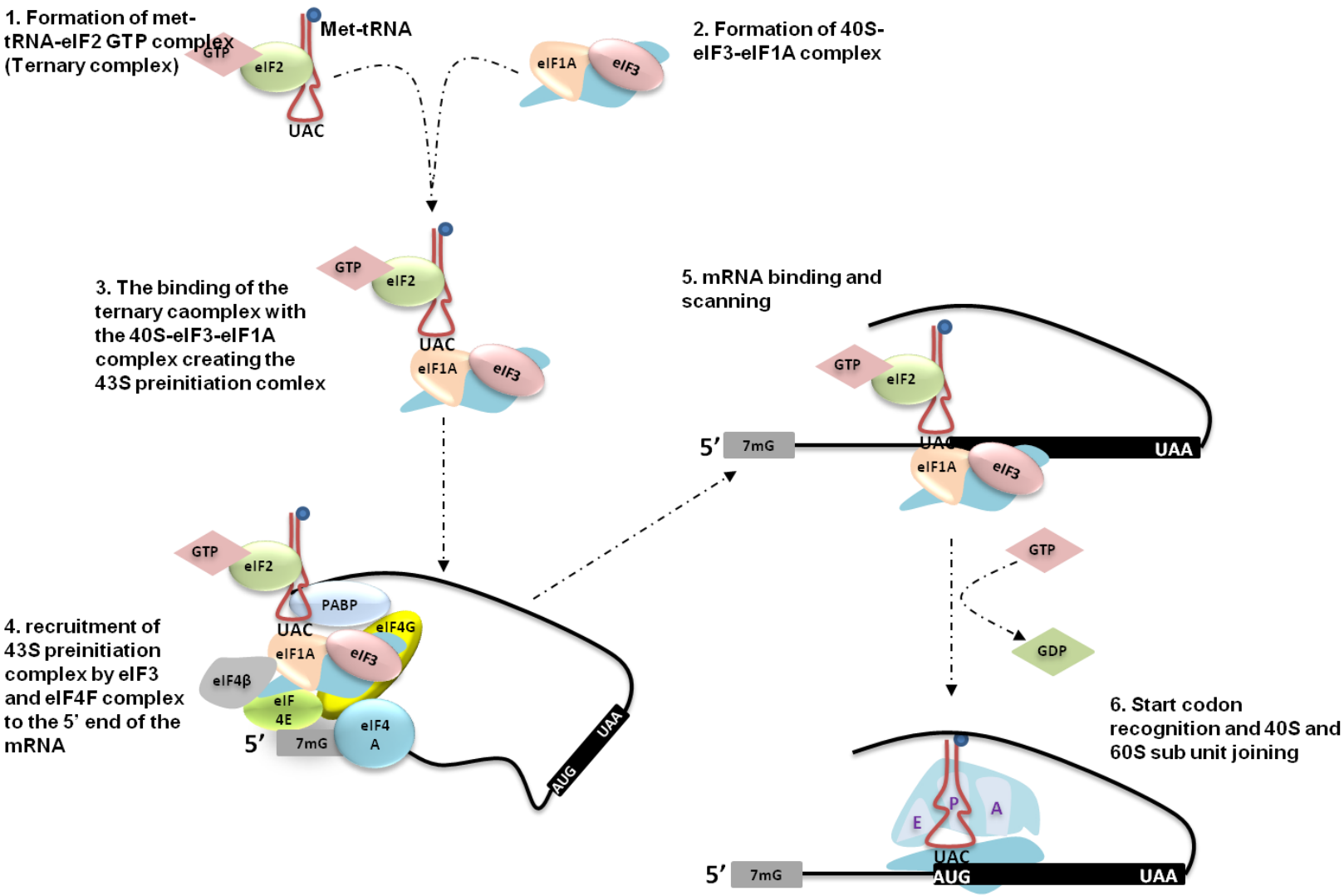


Figure 1.3.1 Cap dependent mRNA translation initiation in eukaryotes (Adapted from Gingras et al., 1999).

40S ribosomal subunit to the 5’ cap of the mRNA. The middle region of eIF4G contains eIF3 binding motif (Pestova et al., 2007). In addition, the N-terminus of eIF4G also contains a PABP binding domain (Imataka et al., 1998).

The 46kDa eIF4A protein is a member of DEAD-box family and possesses RNA dependent ATPase activity and bidirectional RNA helicase activity. The intrinsic ATPase and the helicase activities of eIF4A are very weak. However, it’s ATPase and the helicase activities are strongly stimulated by eIF4B. Nevertheless, the eIF4A and the eIF4B do not directly interact with each other (Andreou and Klostermeier, 2013). The eIF4B interacts with ribosomal 18S rRNA and mRNA (Methot et al., 1996) and is also involved in 48S complex assembly (Pestova et al., 1996b). The ATPase and helicase activity of eIF4A has also been reported to be enhanced by eIF4H. Even though the precise role of eIF4A in unwinding secondary structures in the mRNA is not clear, the most acceptable model on binding of 43S PC to the mRNA is that eIF4F helps in delivering eIF4A to the 5’ end of the mRNA, then eIF4A unwinds the complex secondary structures and produce a single stranded RNA, which allows 43S PC recruitment (Andeou and Klostermeier, 2013).

1.3.3. mRNA circularization during translation initiation

The circularization of mRNA is a central feature for eukaryotic mRNA translation initiation, which acts as a proof reading mechanism ensuring that only properly processed and intact mRNAs are translated. Besides, circularization of mRNA enhances efficient recycling of translation factors and also helps in the stabilization of the mRNA (Weill et al., 2012). The circularization of mRNAs is facilitated by the interaction of the cap with the PABP, eIF4G and eIF4E proteins (Weill et al., 2012). Except histones, all eukaryotic cellular mRNAs are post

transcriptionally modified by the addition of a 3' poly (A) tail, which is involved in mRNA translation initiation activation (Jacobson, 1996). It functions interdependently with the 5' cap to induce a synergistic enhancement of translation (Eckmann et al., 2010). In addition to eIF4E binding domain, eIF4G contains a conserved PABP binding site, Pab1p. Additionally, Pab1p and eIF4E also contains putative binding sites for eIF3 and RNA (Gross and Kleimann, 2013; Kahvejian et al., 2005). The eIF4E-eIF4G-Pab1p complex confers high affinity for the entry of 40S ribosomal subunit and may also stimulate the activity of the other translation initiation factors like eIF4A (Wells et al., 1998). In addition, PABP joins the 40S and 60S ribosomal subunits, stabilizes the eIF4F-cap complex and induces the association of the PIC to the 5' end of the mRNA (Kahvejian et al., 2005).

1.4. mRNA TRANSLATION IN VIRUSES

Both RNA and DNA viruses are totally dependent on the host translational machinery for viral protein synthesis. The ensuing viral protein synthesis is essential for viral genome replication and progeny virus production. For viral mRNA translation initiation to occur, viruses need to recruit cellular ribosomes to their mRNAs. Almost all viruses have developed a strategy to hijack the cellular translation initiation factors and signaling pathways that control the host translation machinery.

1.4.1. Cap mediated translation

In eukaryotic cell co-transcriptional event, a cap structure is added to the nascent 5'-triphosphate of a mRNA in the nucleus that ensures efficient translation of the mRNAs in the cytoplasm. One of the benefit of mRNA capping in eukaryotes is to identify foreign RNAs

including viral transcripts; however, viruses have developed a strategy to protect their mRNAs from the host innate immune system either by adding a cap moiety (7-methyl-Gppp, p is a phosphate group) on their 5' ends that is indistinguishable from cellular mRNA cap structures or by covalently attaching a peptide at the 5' end of the viral mRNAs. In addition to helping escape from the innate immune surveillance, capping confers efficient translation of viral mRNAs using the host translation machinery (Decroly et al., 2012). As compared to eukaryotic mRNA capping, capping of viral mRNAs is diverse in terms of the genetic components, the protein domain organization, the enzyme structure and the reaction mechanisms (Decroly et al., 2012).

Viruses like *Reoviridae* and *Poxviridae* use a RNA capping pathway that is similar to the eukaryotic cells. These viruses appear to encode their own RNA capping machinery, which led to the discovery of RNA cap (Ferron et al., 2012). The conventional eukaryotic mRNA capping mechanism involves hydrolysis of the 5'-triphosphate by an RNA 5' phosphatase (RTPase) to yield 5′ ppN, followed by the addition of cap structure by the capping enzyme (guanylyltransferase [GTase]) by transfering G*p* from a G*p*pp donor to form the cap structure, G*p*ppN. Then, the cap is methylated by a RNA cap guanine N-7-methyltransferase (N-7 MTase) by transfering a methyl group from *S*-adenosyl-L-methionine (SAM) to yield 7^{m}GpppN and *S*-adenosyl homocysteine providing a minimal RNA cap chemical structure named cap-0 (m^{7}GpppNp), This methylated cap can then be subsequently methylated by a ribose-2′-O (2′-O) methylase to yield $7^{m}GpppN^{m}$ (Furuichi and Shatkin, 2000), which is recognized by the translation initiation factor eIF4E (Decroly et al., 2012).

Viruses have evolved different viral mRNA capping mechanisms. For instance, most viruses that synthesize their mRNAs using cellular RNA Pol II exploit the cellular capping machinery including RNA viruses that belong to Retroviridae and Bornaviridae (reviewed in

Decroly et al., 2012). Three major human pathogen families, *Arenaviridae*, *Bunyaviridae* and *Orthomyxoviridae* acquire a cap structure from cellular mRNA by a mechanism called 'cap snatching' which involves the removal and transfer (snatching) of the cap from the cellular mRNA to their own pre-mRNA through endonucleolytic cleavage. The stolen cap is then used to prime synthesis of viral RNA by the viral polymerase (Ferron et al., 2012; Decroly et al., 2012). The L protein of *Arenaviridae* and *Bunyaviridae* contains the structural endonuclease domain that is involved in the cap snatching. The viruses with single stranded (ss) RNA genome synthesize their own cap by 'conventional' (the sequential action of RTPase, GTase and MTases) (eg rabies virus) or 'unconventional' mechanisms (e.g. influenza, lassa, hantaan and rift valley viruses) (Ferron et al., 2012). The best characterized conventional viral-RNA capping system is that exemplified by the double stranded DNA virus vaccinia virus. They acquire their cap by a conventional mechanism using a viral encoded multifunctional mRNA cap synthesizing enzyme (D1), which has domains for RTPase, GTase and N7MTase (Cong and Shuman, 1995). In addition double stranded RNA viruses like those in the *Reoviridae* family use similar mechanisms as vaccinia virus (Decroly et al., 2012).

In contrast, some viruses evolved mechanisms to generate viral mRNA caps using 'unconventional' means. For instance, the ss (-) RNA virus vesicular stomatitis virus (VSV) (a prototype of non segmented negative sense RNA viruses) can synthesize their own cap, which is similar to the cellular RNA cap, but use a different mechanism (Ogino and Banerjee, 2007). In these viruses, the capping enzyme transfers the RNA to G*pp* rather than G*p* into the 5' end of RNA (Ogino and Banerjee, 2007). In addition, the VSV mRNA cap methylase activities are unique in that the G-N-7 and 2′-O methylase activities use a single *S*-adenosyl-L-methionine (SAM) binding site (Li et al, 2006). Generally, the L-protein of VSV and others in the

Mononegavirales have RNA-dependent RNA polymerase (RdRp) and RNA cap synthesis activitities (Murphy and Grdzelishvili, 2009). On the other hand, the ss (+) RNA alphaviruses of *Togaviridae* like Sindbis and Similki forest virus synthesize a cap only up to a cap-0 structure via a non conventional mechanism. The Nsp1 protein encoded by these viruses has guanine-7-methyltransferase activity (Mi and Stollar, 1991; Laakkonen et al., 1994). Furthermore, Ahola and Kaariainen, 1995 reported that the viral mRNA cap complex in these viruses consists of 7-methyl-GMP (m7GMP) linked to nsPl, which is unique to alphaviruses in mRNA cap formation, where the methylation of the guanine precedes the bonding with the 5' end of the RNA. Interestingly, some other viruses like members of *Caliciviridae family*, covalently attach viral encoded peptides at their 5' ends, which directly binds to eIF4E and initiates the viral mRNA translation (Goodfellow et al., 2005).

1.4.2. Internal ribosome entry site (IRES)

The IRES is a highly specialized RNA sequence which is present at the 5'-end of certain viral genomes and functions in recruiting ribosomes to the mRNA in a cap-independent mechanism. First discovered in picornaviruses (Jang et al., 1988; Pelletier and Sonenberg, 1988), IRESs are highly structured and can promote end-independent 40S ribosomal entry without the need for free the 5' ends in IRES containing mRNAs (Hellen and Sarnow, 2001) (Figure 1.4.1).

The IRESs are classified into four structural groups; Type 1 (poliovirus), Type 2 (EMCV), Type 3 (hepatitis C virus) and Type 4 IRESs (cricket paralysis virus) (Kieft, 2008). The classification is based on IRES's secondary structures, the ability of the IRES to operate in rabbit reticulocyte lysate with or without supplementation, the requirement for the eIFs and the

location of the start codon relative to the start of recruitment of the 40S ribosomal subunit (Kieft, 2008).

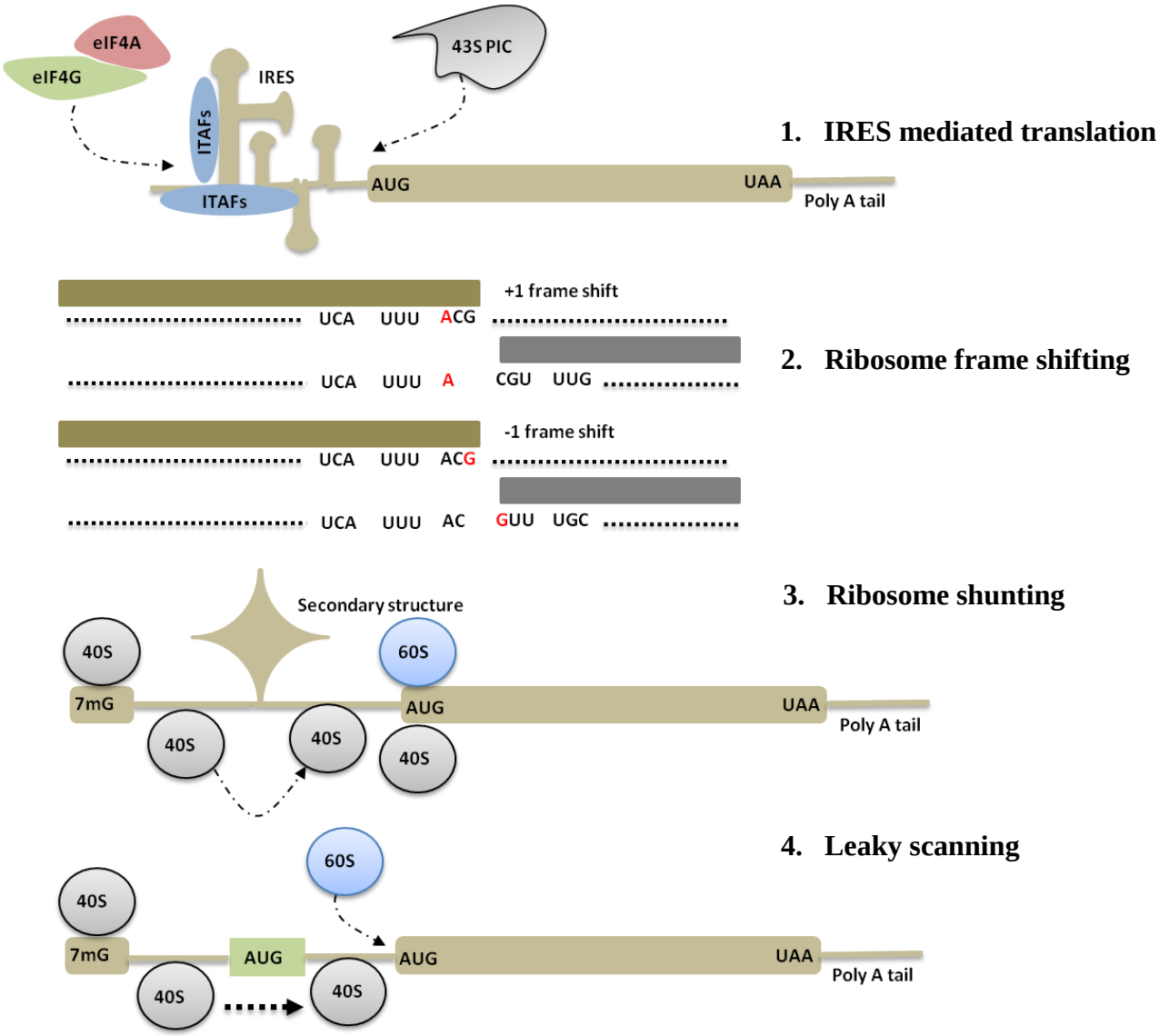


Figure 1.4.1 Schematic of different types of mRNA translation initiation (Adapted from Lopez-Lastra et al., 2005).

In encephalomyocarditits virus (EMCV) IRES dependent mRNA translation, except for eIF4E and the N-terminal domain of eIF4G to which eIF4E binds, all sets of eIFs that are essential for cap dependent translation are required (Pestova et al. 1996a; Pestova et al., 1996b). In EMCV, the domain of the IRES (i.e. type 2 IRES) close to the initiation codon is bound by eIF4G, followed by recruitment of eIF4A and binding of the 43S preinitiation complex to IRES (Kolupaeva et al., 2003).

The mechanism of recruitment of eIFs and ribosome to the mRNA is similar in Type 1 and 2 IRESs (de Breyne et al., 2009). The sequences and secondary structures of type 3 and type 4 IRESs are quite distinct from each other and with that of type 1 and type 2 IRESs. Both Type 3 and 4 IRESs involve eIF independent interaction with the 40S ribosome and influence the entry, positioning and fixation of mRNA in the ribosomal decoding channel by inducing conformational changes in the ribosome (Hellen, 2009).

1.4.3. Frame shifting

Frameshifting is a change or shift in the ribosomes frame by one or two nucleotides during translation (Figure 1.4.1). Generally, programmed frame shifting occurs at shift prone sites in response to stimulatory signals within the mRNA. In response to those signals, ribosomes are induced to move into the -1 reading frame in a 5' direction and start translation in the new reading frame (Dinman, 2012). The cis acting signals that are essential for ribosomal frame shifting are well characterized. However, the mechanism is not well understood (Brierley, 1995). Frame shift signals have been identified from several double and single stranded RNA viral families including *Astroviridae, Luteoviridae, Retroviridae, Togaviridae, Totiviridae, and*

Coronaviridae (Brierley, 1995). In Rous sarcoma virus (RSV), the gag-pro, gag-pol or pro-pol mRNA overlaping regions contain heptanucleotide stretch with homopolymeric triplets (XXXYYYZ). Based on this observation, a simultaneous slippage model of frame shifting was proposed that suggests two adjacent ribosome-bound tRNAs in the zero reading frame (X-XXY-YYN) slip back simultaneously by one nucleotide at the time of the frame shift so that both tRNAs are in the -1 phase (XXX-YYY) and are base paired to the mRNA in at least two out of three anti-codon positions (Dinman, 2012). In addition, efficient frame shifting requires a downstream structure called RNA pseudo-knot, which is formed when nucleotides in the loop region of a hairpin-loop base pair with other regions in the mRNA (Giedroc and Cornish, 2009). Replacing the pseudo-knots with other stable RNA structures significantly reduce the efficiency of ribosomal frame shifting (Brierley et al., 1991; Chen et al., 1995). Mostly, the distance between the slippery sequence and the downstream stimulator appears to be limited to only 5 and 8 nucleotides (Brierley et al., 1992; Kollmus et al., 1994). The degree and the extent of involvement of viral or cellular factors in the ribosomal frame shifting during the course of virus infection is not yet well elucidated (Dinman, 2006).

1.4.4. Leaky scanning

Leaky scanning is a mechanism of translation initiation by which ribosomes bypass the first AUG codon and initiate instead at a second or, sometimes, third AUG codon (Figure 1.4.1). This has an advantage of producing multiple proteins from one mRNA (Kozak, 1991a). Leaky scanning may also occur when initiation happens at a non AUG codon, such as CUG, ACG or GUG (Kozak, 1991b). Several viruses including human immunodeficiency virus (HIV) employ leaky scanning mechanism and economise on their coding space. For instance, the envelope

glycoprotein (ENV) of HIV-1 is translated by leaky scanning and relies on suboptimal translation initiation codons at the upstream rev or vpu AUGs (Schwartz et al., 1990). The E7 oncoprotein of human papilloma virus (HPV) type 16 is translated by a leaky scanning mechanism from E6/E7 bicistronic mRNA (Stacey et al., 2000). In addition, the rabies virus phosphoprotein (Chenik et al., 1995) and certain bicitronic mRNAs of severe acute respiratory syndrome corona virus (SARS-CoV) also produce protein via leaky scanning mechanism (Schaecher et al., 2007; Xu et al., 2009; Yang et al., 2009).

1.4.5. Ribosome shunting

Ribosome shunting is a mechanism by which the scanning 40S ribosomal subunit physically bypasses (shunts) a hairpin loop and strong secondary structures in the 5' untranslated region that would block the scanning process, thereby preserving the secondary structure of the shunted region (Lopez-Lastra et al., 2010) (Figure 1.4.1). Shunting was first characterized in plant retroviruses namely cauliflower mosaic virus (CaMV) 35S RNA (Futterer et al., 1993) and then in Rice tungro bacilliform virus (RTBV) (Futterer et al., 1996). Subsequently, it was observed in other viruses including HPV (Remm et al., 1999), duck hepatitis B pararetrovirus (Sen et al., 2004), Sendai paramyxovirus (de Breyne et al., 2003) and adenovirus late mRNAs (Yueh and Schneider, 1996; Yueh and Schneider, 2000). All adenovirus late mRNAs contain an identical 5' non-coding segment called the tripartite leader (TPL) (an extensive unstructured 5' end, followed by a group of stable hairpin structures) which acts as a *cis*-acting shunting element (Yueh and Schneider, 1996; Yueh and Schneider, 2000). Host cellular protein synthesis is inhibited during adenovirus infection; however, the TPL and 100K protein of adenoviruses form a complex and enhance association of eIF4G and PABP. By doing so, they facilitate the selective

translation of late viral mRNAs by promoting ribosome shunting (Xi et al., 2004; Xi et al., 2005).

1.5. INHIBITION OF mRNA TRANSLATION BY VIRUSES

Viruses are dependent on host translation machinery for translation of viral mRNAs. Regardless of the mechanism used to produce mRNA, viruses recruit cellular translation machinery to the viral mRNAs. Hence, one of the key features during host-cell infection by several viruses is the inhibition of cellular protein synthesis (Walsh and Mohr, 2011). Viruses use different mechanisms to inhibit host cellular mRNA translation. For example, certain viruses inactivate the eIF4F subunits, manipulate the eIF4F binding proteins or remove key structures like m^7G cap from the cellular mRNAs (Schneider and Mohr, 2003). For example, poxvirus decapping enzyme (D9 and D10 protein) inhibits cellular protein synthesis by removing the m^7G from the cellular mRNAs, however, robust transcription of viral mRNAs allow stage-specifc translation of viral proteins (Parrish et al., 2007). VPg protein of calciviruses directly binds eIF4E and recruits eIFs to viral mRNA inhibiting cellular mRNA translation. Vesicular stomatitis virus VSV and SV40 inhibit host protein synthesis by dephosphorylating the 4EBP1, which further activates the eIF4E. The 2A protease of enteroviruses and 3C protein of calciviruses cleave eIF4G. In addition, 3C protease of enteroviruses cleaves eIF5B and PABP leading to the suppression of global cellular protein synthesis. Other viral gene products interfere with cellular mRNA translation by directly binding with initiation factors. For example, capsid protein of rubella virus directly binds PABP and suppresses the host mRNA translation. The N protein of measles virus, M protein of Rabies virus and spike protein of SARS CoV bind with eIF3 and impair cellular mRNA translation (Walsh and Mohr, 2011).

1.6. ADENOVIRUS INHIBITION OF HOST CELLULAR PROTEIN SYNTHESIS

Adenoviruses inhibit cellular protein synthesis by inhibiting translation of cellular capped mRNAs, especially during late times post infection (Dolph et al., 1988; Huang and Schneider, 1991). Although the cellular mRNA translation is blocked during adenovirus infection, little is known about the identity of the proteins (viral or cellular) and the mechanisms involved in the process (Yueh and Schneider, 1996). Cellular mRNA translation in adenovirus infected cells is diminished by infection induced displacement of Mnk1 from eIF4G via direct binding of adenovirus 100K protein to eIF4G causing under-phosphorylation of eIF4E. However, only four fold reduction in host cellular mRNA translation was observed in adenovirus infected cells overexpressing a phosphorylation mutant of eIF4E suggesting that underphosphorylation of eIF4E is not the only mechanism in the process of overall suppression of global cellular protein synthesis in adenoviruses infected cells (Cuesta et al., 2004).

1.7.DEAD BOX RNA HELICASES

DExD/H helicase family of proteins which includes DEAD-box proteins and the related DEAH, DExH and DExD proteins are a closely-related family of proteins, which can be identified based on the differences in their shared eight conserved motifs (motifs I, Ia, Ib, II, III, IV, V and VI) (de la Cruz et al., 1999). They are classified under super family 2 (SF2) helicases. Based on the structural and the functional studies, the DEAD box family is known to be the largest family of RNA helicases composed of nine motifs (Figure 1.7.1). Motifs Q, I and II are involved in the nucleotide binding; Motifs Ia, Ib, IV and V are involved in RNA binding; Motifs III and possibly VI are involved in ATP hydrolysis (Reviewed in Hogbom et al., 2007) (Figure 1.7.1). Motif II, which is also known as the Walker B motif contains the amino acid sequence

Figure 1.7.1 The different motifs of the DEAD box family of RNA helicases (Adapted from Cordin et al., 2006).

D-E-A-D (asp-glu-ala-asp) from which the name DEAD box protein is derived. Each of the motifs of DEAD-box proteins has distinct structure and function. In addition, the DEAD-box proteins are present in all eukaryotic cells and in most prokaryotes like eubacteria and archaebacteria, and involve in diverse biological processes, especially in almost all steps of RNA homeostasis including transcription, splicing, ribosome biogenesis, export, translation and decay (Figure 1.7.2) (Cordin et al., 2006).

1.7.1. DDX3

The DDX3 is a member of the DEAD box family of ATP dependent RNA helicases, which exists in two closely related forms (92% protein identity) namely DDX3X and DDX3Y. DDX3Y is produced specifically in the spermatocytes and is essential for the spermatogenesis while DDX3X is ubiquitously expressed in a wide variety of tissues and participates is several metabolic processes. DDX3X is a 73kDa nucleo-cytoplamic shuttling protein possessing ATPase and RNA helicase activities.DDX3 is essential for mRNA transcription, pre-mRNA splicing, mRNA export, translation and mRNA turn over (Rocak and Linder, 2004; Kwong et al., 2007) (Figure 1.7.2). It is also implicated in translation initiation (Lee et al., 2008; Parsyan et al., 2011) and may regulate the cellular mRNA translation by interacting with specific eIFs like eIF4E and PABP (Shih et al., 2008, Shih et al., 2012), eIF2α (Lai et al., 2008), and eIF3 (Lee et al., 2008). It can also act as a scaffold for alternative protein-protein interactions. Moreover, DDX3 has also been shown to be involved in the regulation of innate immunity against microbial infections (Oshiumi et al., 2010).

The interaction of viral proteins with cellular proteins is of vital importance in the regulation of virus replication, growth and survival. In recent years, viral proteins encoded by

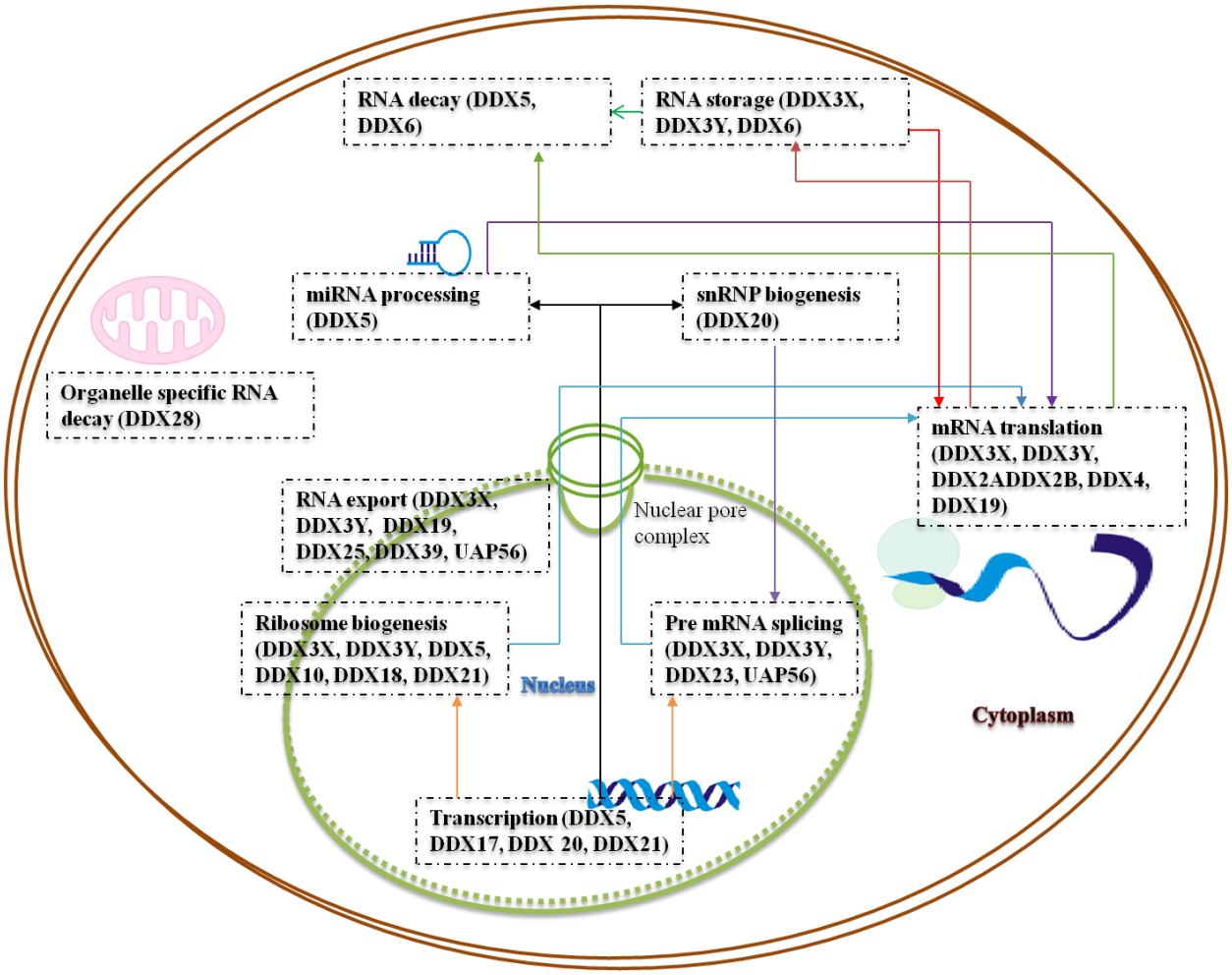


Figure 1.7.2 Functions of DEAD box family of RNA helicases (Adapted from Linder and Jankowsky, 2011)**.**

vaccinia virus (Schroder et al., 2008), hepatitis B virus (Wang and Ryu, 2010; Yu et al., 2010), hepatitis C virus (Ariumi et al., 2007; Owsianka and Patel, 1999) and HIV (Yedavalli et al., 2004) have been shown to interact with DDX3, which appears to be required for the efficient replication of their viral genomes. The viruses accomplish this either by exploiting or suppressing the functions of DDX3.

1.7.1.1.The role of DDX3 in DNA transcription

The DEAD box proteins have been reported to interact with the transcription machinery without employing their helicase or ATPase activity. Studies suggest that DDX3 may be involved in the regulation of transcription. For instance, DDX3 up regulates the activity of the INFβ promoter through TBK-1 and IKK-e mediated INFβ promoter induction (Schroder et al., 2008). Oshiumi et al., 2010 also showed that the C-terminal (622-662 a.a.) part of DDX3 interacts with IPS1 (IFN-β promoter stimulator-1) also known as MAVS (mitochondrial anti viral signaling) and colocalize in the membrane of the mitochondria, which is important in the enhancement of the MAVS mediated INF-β promoter activation. On the contrary, while DDX3 forms a complex with E-cadherin promoter and transcriptionally represses the expression of E-cadherin, the down regulation of DDX3 promotes the transcription of E-cadherin (Botlagunta et al., 2008). DDX3 also induced a 2- to 4-fold up-regulation of $p21^{waf1/cip1}$ promoter–driven luciferase activity. This effect of DDX3 is caused by its binding to the transcription factor Sp1, which requires the ATPase, but not the helicase activity of DDX3 (Chao et al., 2006). The mechanisms of recruitment of DDX3 to the promoter sites are not well understood. Other members of the DEAD-box family of proteins are also shown to be involved in transcriptional regulation. DDX54 (DP97) enhances the activity of the promoter of the nuclear receptor estrogen

receptor-α (ERα) by interacting with the AF2 domain (i.e. transcription activation domain) of ERα and other nuclear receptors (Rajendran et al., 2003). DDX5 (P68), another member of DEAD-box family of proteins mediates interactions between ERα, AF1 and the AF2 co-activator complex and act as a transcriptional co-activator for ERα transcription by acting as a bridge between the transcription factors (Watanabe et al., 2001). There is also a strong evidence that RNA helicase-A, a member of the DEAD box proteins acts as a bridging factor between the CREB-binding protein (CBP) and RNA polymerase-II, hence cooperating with CREB to activate transcription (reviewed in Fuller-Pace, 2006).

1.7.1.2.The role of DDX3 in pre-mRNA splicing

Splicing is a step at which the introns are removed from pre-mRNAs and exons are joined together to produce a continuous reading frame. Pre-mRNA splicing is a multistep process that involves ATP, two trans-esterification reactions and structural rearrangement of the spliceosome, a dynamic RNA-protein complex in which splicing takes place. The spliceosome is composed of snRNPs (U1, U2, U4/U6 and U5 spliceosomal snRNPs) as well as non-snRNP proteins. The DDX3 protein contains a region that resembles the RS-domains in splicing factors like ASF/SF2 and SC-35, a domianinvolved in mediating protein-protein interactions (Kohtz et al., 1994; Wu and Maniatis, 1993). DDX3 and its yeast homologue ded1p were also isolated as a component of the spliceosome, which remain associated with mature mRNA after the splicing stage in an Exon junction complex (EJC) dependent manner. However, it still is unclear whether DDX3 is functionally involved in the process of splicing or not.

1.7.1.3. The role of DDX3 in mRNA export

Since mRNA transcription and translation occur at different cellular compartments, the mature mRNAs after post ttranscriptional modification needs to be exported from nucleus to the cytoplasm by various nuclear export receptors. The two well characterized proteins that function as nuclear export receptor are CRM1, which exports proteins that contain a leucine-rich nuclear export signal (Katharine et al., 1997), ribosomal RNAs and small nuclear RNAs, and the Tip associated protein (TAP) which is mainly involved in exporting mature mRNAs (Katahira et al., 1999; Lai et al., 2008). Reports suggest that DDX3 interacts with both CRM1 and TAP (Dayton, 2004; Lai et al., 2008). The C-terminus of DDX3 is responsible for its interaction with TAP, in the main mRNA export pathway. Despite the evidence that DDX3 interacts with proteins in the mRNA export pathways, nobody has yet shown the specific roles of DDX3 in export of mature mRNAs (reviewed in Schroder, 2010).

1.7.1.4.The role of DDX3 in mRNA translation

DDX3 is implicated in translation initiation and may regulate the cellular mRNA translation by interacting with specific translation initiation factors eIF4A, eIF4E, eIF2α, PABP and eIF3. Though few studies indicate the role of DDX3 in the repression of cap-dependent translation initiation (Shih et al., 2008), recent data suggests the involvement of DDX3 in enhancing translation initiation (Hilliker et al., 2011; Geissler et al., 2012; Soto-Rifo et al., 2012). The translation of β-globin mRNA was significantly reduced in DDX3 knocked down Hela cells transfected with plasmid DNA expressing β-globin under the control of CMV promoter (Lee et al., 2008). DDX3 also may bind to poly $(A)^+$ RNAs both in the cytoplasm and the nucleus independent of TAP (Lai et al., 2008). Immunoprecipitation experiments show that

DDX3 interacts with eIF4A, eIF2α and PABP1 and the 40S ribosome subunit in enriched fractions of HEK293 cytoplasmic extracts. Moreover, DDX3 gene silencing results in the suppression of the translation of mRNAs containing complex 5'secondary structures suggesting the direct involvement of DDX3 in the translation process (Lai et al., 2008). On the other hand, Soto-Rifo et al., 2012 showed that DDX3 non-specifically binds to the 5' end of mRNAs and clamps the binding of 5' cap-binding complex, eIF4F. DDX3 interacts with the eIF4F complex in addition to eIF4B and poly A binding protein (PABP). Similar results have also been reported with the yeast homologue of DDX3, Ded1 (Hilliker et al., 2011). DDX3 knockdown or overexpression had a negative and positive impact, respectively on the translation of different reporter mRNAs indicating a general role of DDX3 in promoting protein translation (Geissler et al., 2012). In addition, Geissler et al., 2012 reported an RNA independent interaction of DDX3 with ribosomal protein S6 (rpS6), a component of the 40S subunit of the ribosome and with an RNA binding protein, IGF2BP1/2. They have also demonstrated that DDX3 is part of 43S and 80S ribosomal pre-initiation complexes and help in the assembly of functional 80S ribosomes. These results suggested that DDX3 is not required for the elongation or termination phase of the translation process, but important for the translation initiation process up to the assembly of functional 80S ribosome.

Similarly, the yeast homologues of DDX3, Ded1p and Dbp2 are reported to be indispensable translation initiation factors in yeast. They are suggested to be involved in the unwinding of the 5' untranslated regions in concert with eIF4A (Tarn and Chang, 2009). Another study shows the function of Ded1 in the scanning process by testing different mRNAs with 5'-untranslated regions of different lengths (Berthelot et al., 2004).

1.7.1.5.The role of DDX3 in innate immunity

RNA helicases play a significant role in the innate immunity against viral infections. Toll like receptor (TLR)-independent pattern recognition receptors (PRRs), like retinoic acid inducible gene-I (RIG-I/DDX58) and melanoma differentiation-associated gene 5 (Mda5) that are expressed in nucleated cells contribute significantly in mounting an innate immune response against pathogens. Both RIG-I and Mda5, classified as RIG-I like DExH family members, contain two N-terminal caspase recruitment domains (CARDs) and a DExD/H box RNA helicase domain. RIG-I/Mda5 use the mitochondrial CARD containing adaptor protein MAVS/IPS-1 that helps for linking detection of dsRNA to activation of TBK1 (TANK-binding kinase-1) / IKKϵ (IkB kinase-ϵ) that phosphorylates the transcription factors IRF-3/IRF-7 which will then be translocated into the nucleus and activate the production of type I INF (Yoneyama et al., 2004). DDX3 promotes innate immunity by transiently interacting with IKKϵ and TBK-1. DDX3 may also interact with RIG-I, Mda5 and the CARD domain of MAVS, and act upstream of IKKϵ and TBK1 in the MAVS dependent signaling pathway. In addition, it has been reported that DDX3 is also involved in the TRIF (TIR domain containing adaptor inducing interferon-β) dependent TLR3/4 immune signaling pathways. However, the exact mechanism of action in all the indicated innate immune signaling pathways is yet to be determined (reviewed in Schroder, 2010).

Despite the fact that several studies show the multifunctional property of DDX3, the exact mechanisms employed by DDX3 in the different cellular processes that it participates in is not yet fully elucidated.

1.7.1.6. Manipulation of DDX3 by viruses

It is clear that DDX3 participates in two basic biological processes namely RNA metabolism and innate immunity. Most of the studies indicate a positive role of DDX3 in innate immunity and RNA metabolism (mRNA synthesis to mRNA translation). Both processes are required by viruses since most lack the basic elements needed to complete the virus life cycle, from DNA replication to protein synthesis and are completely dependent on the host molecular machinery, most viruses also face a challenge from the host immune system during infection and must devise strategies to negatively regulate the immune system at different levels. Since DDX3 appears to be involved in a number of cellular processes, the manipulation of DDX3 by specific viral products is attractive for the purpose of supporting viral replication and evasion from the immune system.

The first virus that was identified to have a gene product that targets DDX3 was Hepatitis C virus (HCV) (Owsianka and Patel, 1999). Owsianka and Patel, 1999 demonstrated that the Core protein of HCV directly interacts with the C-terminal region (aa 553-622) of DDX3 and changes the normal cellular localization of DDX3 into distinct spots in the cytoplasm co-localizing with the core protein. Interaction of HCV Core protein with DDX3 inhibits translation of capped but not uncapped mRNAs in *in-vitro* reticulocyte lysate assay which was presumed to be a result of the inhibition of the function of DDX3 as an RNA helicase (Mamiya and Worman, 1999). In addition, knockdown of DDX3 shuts off the replication of HCV suggesting the association of DDX3 with HCV replication complexes or incorporation of DDX3 into the HCV virion through interaction with the core to act as an RNA chaperone (Ariumi et al., 2007). This reasoning is based on a previous work that demonstrated the detection of DDX3 in the lipid droplets of core-expressing Hep39 cells through proteomic analysis (Sato et al., 2006).

Interestingly, infection of the cells with HCV does not normally bring about a difference in the DDX3 protein level. The exact mechanism by which the interaction of HCV and DDX3 enhances the replication of the viral genome is still unexplained (Ariumi et al., 2007).

DDX3 appears important for replication of HIV. The expression of DDX3 was enhanced in a Tat dependent manner in HIV infected cells. The Rev protein of HIV and the cellular CRM1 protein interact with DDX3 and mediate nuclear export of the spliced viral mRNA through the CRM1 dependent pathway (Yedavalli et al., 2004). The Rev-viral mRNA interaction is mediated through the cis-acting Rev Response Element (RRE) of HIV-1 intron containing mRNAs to which Rev protein binds (Malim et al., 1989). It has also been shown that the helicase function of DDX3 is essential for HIV mRNA export (Schroder, 2010). Exogenous expression of DDX3 was shown to increase the expression the HIV Gag protein by 5 to 10 fold, while dominant-negative DDX3 mutant suppressed the expression of Gag (Yedavalli et al., 2004). Moreover, knockdown of DDX3 gene by using anti DDX3 shRNA shuts down the replication of HIV without affecting cell viability (Ishaq et al., 2008). Thus, DDX3 is considered a potential target for the treatment of HIV infections because of its crucial involvement in the fulfillment of the virus' life cycle (Yedavalli et al., 2004).

DDX3 is also targeted by K7 protein of vaccinia virus (Schroder et al., 2008) and hepatitis B virus polymerase (HBV Pol) protein (Wang et al., 2009). These viruses inhibit the function of DDX3 in innate immunity. K7 protein of vaccinia virus associates with the N-terminal domain of DDX3 and prevents the association of DDX3 and IKKϵ, subsequently preventing the IKKϵ from activation of downstream transcription factors that induce the production of interferons (Schroder et al., 2008). DDX3 inhibits replication of HBV by interacting with viral Pol in an RNA independent manner that interferes with the reverse

transcription activity of the virus. The exact mechanism of interference is not known. Interestingly, it was reported that HBV down regulates the expression of DDX3 in HBV-induced hepatocellular carcinoma. This might be to alleviate the inhibitory effect of DDX-3 to promote its own life cycle (Wang et al., 2009). Similar to K7 protein of vaccinia virus, HBV Pol dampens the interaction between TBK1/IKKϵ and DDX3 and inhibits the RIG-I and TLR-3 mediated interferon induction (Yu et al., 2010).

Recently, Chahar et al., 2012 showed the co-localization of NS3 protein of West Nile virus (WNV) and DDX3 in the peri-nuclear region of infected cells. West Nile virus normally replicates in the peri-nuclear replication complexes, which are invaginations of rough endoplasmic reticulum membrane vesicles. The level of DDX3 in WNV infected cells increased by 2-4 fold. Interestingly, siRNA knockdown of DDX3 significantly reduced the replication of WNV. In murine norovirus infection, DDX3 associates with the viral RNA during replication and the silencing of DDX3 cause 80-90% reduction in the levels of viral NS7 protein, viral yield and viral RNA levels suggesting that DDX3 is essential for the life cycle of norovirus (Vashist et al., 2012).

2. HYPOTHESIS AND OBJECTIVES

Although few reports have evaluated the structure and function of BAdV-3 proteins (reviewed in Russel, 2000; Russel, 2009) there is still a lack of understanding of a number of the molecular mechanisms that function in the infected cell, principally in respect to how and why the virus gene products interact with cellular components. Of all the known adenoviral minor proteins, protein pVIII, which connects the core with the inner surface of the adenovirus capsid, is the least characterized (Martin, 2012). The protein is present as 120 copies /virion (Reddy et al., 1998) and plays a role in the stability of virion structure (Liu et al., 1985). The pVIII synthesized in a precursor form (Takahashi et al., 2006; Vellekamp et al., 2001) has potential adenovirus specific protease cleavage sites (Diouri et al., 1996). The precursor form of pVIII is present in immature virions, while only cleaved form of pVIII is present in mature virions (Chelius et al., 2002; Takahashi et al., 2006). Cleavage of pVIII by adenovirus protease appears essential for maturation of progeny virions (Gastaldelli et al., 2008). High resolution structural studies suggest that pVIII interacts with hexon and IIIa protein (Martin, 2012) in the capsid of human adenovirus.

BAdV-3 pVIII is a minor structural protein that is encoded in the L6 region of the late transcription unit, which also encodes the non-structural proteins 33K and 100K (Reddy et al., 1998). Protein pVIII of BAdV-3 contains 216 amino acids and contains two potential adenovirus specific protease cleavage sites (108IAGG↓G and 143LGGG↓S) (Reddy et al. 1998). Detailed molecular and functional characterization of pVIII and the role it plays in the replication of adenovirus is poorly studied both in human and non-human adenoviruses. Recently, using yeast II hybrid assay, we confirmed the interaction of pVIII of BAdV-3 with the cellular DDX3 protein which is mainly involved in the processing of mRNA.

We hypothesize that BAdV3 pVIII interacts with the cellular helicase DDX3 and enhances the life cycle of BAdV-3 at late times post infection by modulating eukaryotic initiation factors which leads to the inhibition of the translation of capped cellular mRNA and augmentation of viral late mRNA translation.

The specific objectives of this work are:

1. Characterization of polypeptide VIII of BAdV-3

2. Confirmation of interaction of pVIII with DDX3

3. Study the biological significance of the interaction of pVIII with DDX3

The following Sections contain details of the findings of the study.

3. CONSERVED REGIONS OF BOVINE ADENOVIRUS-3 pVIII CONTAIN FUNCTIONAL DOMAINS INVOLVED IN NUCLEAR LOCALIZATION AND PACKAGING IN MATURE INFECTIOUS VIRION

(Ayalew LE, Gaba A, Kumar P, Tikoo SK. 2014, Journal of General Virology doi: 10.1099/vir.0.065763-0)

Authors contributions: LEA performed all the experiments and wrote the manuscript, AG helped with creating mutant pVIII, PK helped in performing and analyzing con-focal microscopy and SKT helped in designing the experiments and proof read the manuscript.

3.1. INTRODUCTION

Adenoviruses are non enveloped DNA viruses with icosahedral capsid virions composed of major (hexon, penton, fiber) and minor (pIIIa, pVI, pVIII and pIX) structural proteins surrounding the core. The adenovirus core contains the genome (McConell & Imperiale, 2004; Nemerow et al., 2009) and core proteins (V, VII, mu and terminal protein [TP]) that directly bind the viral DNA. The proteolytic cleavage of some structural proteins including pVIII by adenovirus encoded protease (Anderson et al., 1973; Tremblay et al., 1983; Webster, 1989) is required for maturation of newly formed virions. Assembly of adenoviruses occurs in the nucleus followed by viral DNA replication and late gene expression. Capsid formation is facilitated by the trimerization of hexon protein using 100K, 33K and IVa2 proteins as scaffolding proteins. The fiber trimers then interact with the penton base and assemble with hexon forming an intact capsid. 52K and IVa2 viral protein interact with the packaging domain of the viral DNA and function in DNA encapsidation (Ostapchuk and Hearing, 2005).

Since assembly of adenovirus capsid takes place in the nucleus, structural proteins synthesized in the cytoplasm are transported to the nucleus. Migration of macromolecules from the cytoplasm to the nucleoplasm occurs through the nuclear pore complex (NPC) (Adam, 1999; Kohler et al., 1999). The NPC is composed of 50 – 100 different species of proteins called nucleoporins with an estimated total mass of ~125MDa in higher eukaryotes. NPCs are incorporated into the nuclear envelope at sites where the inner and outer nuclear membranes are fused. They function as gate keepers of the nucleus, performing the essential cellular role of mediating an exchange of proteins, nucleic acids, and other factors between the cytoplasm and nucleoplasm (Theerthagiri et al., 2010).

The NPC permits a non-selective passive diffusion of molecules smaller than 40kDa through the nuclear envelope via its aqueous channel of ~10nm diameter (Paine et al., 1975). NPCs also mediate selective active transport of particles as large as 25nm in diameter or several million Daltons in molecular weight (Feldherr et al., 1984). Directed transport requires energy and soluble factors and is triggered by short strings of amino acids called nuclear localization signals (NLS) on the protein to be transported into the nucleus (Davis, 1995; Gorlich & Mattaj, 1996). The classical NLS, designated as basic type NLS, was first identified in nucleoplasmin (KRPAATKKAGQAKKKK) and the SV40 large T antigen (PKKKRKV) (Dingwall & Laskey, 1991). However, other type NLS including non classical type have been identified (Christophe et al., 2000).

The L6 region of the late transcription unit of bovine adenovirus (BAdV)-3 encodes two non structural (33k, 100k) and one structural (pVIII) protein (Reddy *et al.,* 1998). In an effort to determine the characterstics and functions of the L6 region proteins, we demonstrated that BAdV-3 33K interacts with 100K (Kulshrestha & Tikoo, 2008) and is involved in viral capsid assembly by promoting an efficient interaction between the capsid protein and the viral DNA (Kulshreshtha et al., 2004) and recently demonstrated that cleavage of BAdV-3 100K is not essential for the virus' life cycle (Makadiya, 2013). To further advance our understanding of the BAdV L6 proteins we have identified that BAdV-3 pVIII, a minor capsid protein of 216 amino acids is processed at two canonical adenovirus protease specific cleavage sites (108IAGG↓G and 143LGGG↓S) (Reddy et al., 1998). We also identify the domains of pVIII involved in nuclear localization and incorporation in mature virions.

3.2.MATERIALS AND METHODS

3.2.1. Cell lines and Viruses

Madin Darby Bovine Kidney (MDBK) cells were grown in minimum essential medium (MEM; Sigma Aldrich) containing 5% fetal bovine serum and 50µg/ml gentamycin. African green monkey kidney (Vero) cells were grown in Dulbecco's Modified Eagle's (DMEM; Sigma Aldrich) medium containing 10% heat inactivated fetal bovine serum (Sera Care Life Sciences, Inc.) and 50µg/ml gentamycin. For BAdV-3 purification, confluent MDBK cells grown in 150mm flasks were infected with wild -type BAdV-3 at an MOI of 5. Immature and mature BAdV-3 virions were purified by double Cesium chloride density gradient centrifugation as described earlier (Tollefson et al., 1998). The purified mature virions and empty capsids were stored at -70^{o}c in PBS containing 10% glycerol.

3.2.2. Peptide synthesis and production of polyclonal antibodies

To make antibodies against pVIII, rabbits were immunized with the individual conjugated peptides (500µg/rabbit) emulsified with Freund's Complete Adjuvant (FCA) followed by two injections (conjugated peptide, 300 µg/rabbit) in Freund's incomplete adjuvant (FIA) at four weeks apart. The peptides contained amino acids 85 to 109 (VIIIa) and 187-216 (VIIIb) of pVIII and were synthesized based on intrinsic prediction of hydrophilicity of amino acid side chains using the Pioneer Peptide Synthesis system (Perkin Elmer). The peptides were individually conjugated to keyhole limpet hemocyanin (KLH) as a carrier molecule. Serum was collected ten days after the third injection and was tested for specificity by Western blot.

For *in-vitro* nuclear import blocking experiment, nuclear import protein inhibitory peptides: importin β binding domain of importin α (IBB importin α) (^{10}RMRKFKNKGK

DTAELRRRRVEVSVELRKAKKDEQILKRRNVC [51)]) (Gorlich et al., 1996a), Ycbp80 ([1]MFNRKRRGDFDED ENYRDFRPRMPKRQRIP[30]) (Gorlich et al., 1996b) and SR1 (CGGGRKRRQRSRSRSRSRSR SRSRSRRRR) (Lai et al., 2001) were synthesized using Pioneer Peptide Synthesis system (Perkin Elmer).

3.2.3. Construction of expression vectors

Plasmids encoding glutathione-S-transferase (GST) fused importin α-1, importin α-3, importin α-5, importin α-7 and importin β (kindly provided by Dr. M. Köhler) have been described (Depping et al., 2008). Plasmids GST-NLS-GFP, (GST fused to NLS of simian virus 40 (SV40) T antigen) GST-RanQ69L (dominant negative mutant of Ran fused to GST) were kindly provided by Dr. Y. Yoneda (Tachibana et al., 2000). Plasmid pc3HA (pcDNA3 containing 3 tandem HA tags inserted at *Hind*III-*Ecor*I site) was a gift from Dr. Joyce Wilson, University of Saskatchewan, Canada. The construction of other plasmids is described elsewhere.

a) Plasmid pEY.pVIII. A660bp DNA fragment containing pVIII gene was amplified by using PCR and primers VIII-F and VIII-R and template plasmid pFBAV-302 (Zakarchouk et al., 2004) (Table 3.2.1). The PCR product was digested with *EcoRI* and ligated to *Afe*I and *Eco*RI digested plasmid pEYFPN1 creating plasmid pEY.pVIII.

b) Plasmid pRSET.pVIII. A655bp DNA fragment containing pVIII gene was amplified by PCR using primers *Xho*I VIII F and *Ecor* I VIII R (Table 3.2.1) and plasmid pFBAV302 (Zakarchouk et al 2004) DNA as a template The PCR product was digested with *Xho*I and *Eco*RI and ligated to *Xho*I-*Eco*RI digested plasmid pRSET-A (Invitrogen) creating plasmid pRSET.pVIII.

c) Plasmid GST.pVIII. A 666bp *Hind*III (blunt end repaired by Klenow) – *Xho*I DNA fragment containing pVIII gene was isolated from plasmid pRSET A-pVIII and ligated to 4681bps *Aat*II (blunt end repaired by klenow)) - *Xho*I digested fragment of plasmid pGEX-5X-1 (GE Healthcare) creating plasmid pGST.pVIII.

d) *Plasmid pEY.VIIId1.* A 228bp DNA fragment was amplified by PCR using primers pVIII-D1-F and pVIII-D1-R (Table 3.2.1) and plasmid pEY.pVIII DNA as a template. The PCR product was digested with *NheI* and *EcoRI* and ligated to *NheI* and *EcoRI* digested plasmid pEY.pVIII creating plasmid pEY.VIIId1

e) *Plasmid pEY.VIIId2.* A405bp DNA fragment was amplified by PCR using primers pVIII-D1-F and pVIII-D2-R (Table 3.2.1) and plasmid pEY.pVIII DNA as a template. The PCR product was digested with *NheI* and *EcoRI* and ligated to *NheI* and *EcoRI* digested plasmid pEY.pVIII creating plasmid pEY.VIIId2.

f) Plasmid pEY.VIIId3. A264 DNA fragment was amplified by PCR using primers pVIII-D3-F and pVIII-D3-R (Table 3.2.1) and plasmid pEY.pVIII DNA as a template. The PCR product was digested with *Nhe*I and *Eco*RI and ligated to *Nhe*I and *Eco*RI digested plasmid pEY.pVIII creating plasmid pEY.VIIId3.

g) Plasmid pEY.VIIId4. A 441bp DNA fragment was amplified by PCR using primers pVIII-D4-F and pVIII-D3-R (Table 3.2.1) and plasmid pEY.pVIII DNA as a template. The PCR product was digested with *NheI* and *EcoRI* and ligated to *Nhe*I and *Eco*RI digested plasmid pEY.pVIII creating plasmid pEY.VIIId4

h) *Plasmid pEY.VIIId5.* A 120bp DNA fragment was amplified by PCR using primers pVIII-D5-F and pVIII-D1-R (Table 3.2.1) and plasmid pEY.pVIII DNA as a template. The PCR

product was digested with *Nhe*I and *Eco*RI and ligated to *Nhe*I and *Eco*RI digested plasmid pEY.pVIII creating plasmid pEY.VIIId5.

i) Plasmid *pEY.VIIId6*. A 168bp DNA fragment was amplified by PCR using primers SnaBI-F and EcoRI-R (Table 3.2.1) and plasmid pEY.pVIII DNA as a template. The PCR product was digested with *SnaB*I and *EcoR*I and ligated to *SnaB*I and *EcoR*I digested plasmid pcDNA3.EYFP (Paterson et al., 2012) creating plasmid pEY.VIIId6.

j) Plasmid *pcEY.pVIIId1*. Plasmid pEY.pVIIId1 was digested with *Nhe*I (blunt end repaired with T4 polymerase) and *Xba*I. The 997bp fragment was gel purified and cloned into *Eco*RI (blunt end repaired with T4 polymerase) and *Xba*I digested plasmid pc3HA creating plasmid pcEY.pVIIId1.

k) Plasmid *pcEY.pVIIId4*. Plasmid pEY.pVIIId4 was digested with NheI (blunt end repaired with T4 polymerase) and XbaI. The 1210bp fragment was gel purified and ligated to *Eco*RI (blunt end repaired with T4 polymerase) and *Xba*I digested plasmid pc3HA creating plasmid pcEY.pVIII4.

l) Plasmid pNLS.EYFP. A 833bp DNA fragment was amplified by PCR using primers NLS-EY-HindIII-F and NLS-EY-XbaI-R (Table 3.2.1) and plasmid pVIIId1 DNA as a template. The PCR product was digested with *Hind*III and *Xba*I and ligated to *Hind*III and *Xba*I digested plasmid pcDNA3 creating plasmid pNLS. EYFP.

m) Plasmid *pNLSGFP-βgal*. A 67bp DNA fragment was amplified by PCR using primers NLS-EY-HindIII-F and gbeta-NheI-R (Table 3.2.1) and plasmid pVIIId1 DNA as a template. The PCR product was digested with *Hind*III and *Nhe*I and ligated to *Hind*III and *Nhe*I digested plasmid pCMVGFP-βgal (Wu & Tikoo, 2004) creating plasmid pNLSGFP-βgal.

3.2.4. Mass Spectrometry

Proteins from mature BAdV-3, immature BAdV-3 or lysates of BAdV-3 infected MDBK cells were separated by 10% or 15% sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) or 4 – 15% precast gradient gel (Bio-Rad). The separated proteins were electrophoretically transferred onto Polyvinylidene fluoride (PVDF; Bio-Rad) membrane by wet transfer method. The membranes were then blocked with 10% skimmed milk at +4^{o}c overnight.

Western blots were performed using α-pVIIIa and/or α-pVIIIb polyclonal primary antibodies (1:300) followed by alkaline phophatase conjugated goat anti-rabbit secondary antibody (Jackson Immunoresearch) diluted 1:10,000 or IRdye 680 donkey anti-rabbit IgG (Li-COR). For visualization of protein bands, the SDS-PAGE gels were stained with Coomassie blue for 30 minutes and de-stained with a de-stain solution (40% methanol, 10% acetic acid). For Mass spectrometry analysis, the desired protein bands were cut from the 4-15% precast gradient gel after staining with coomassie brilliant blue and sent for analysis to PBI, Saskatoon.

3.2.5. Confocal microscopy

MDBK cells at 80% confluency in 2 well slides (Thermo scientific) were infected with wild-type BAdV-3 using a Multiplicity of infection (MOI) of 5. At 36hrs post infection, the cells were fixed with 3.7% para formaldehyde for 15min, blocked with 2% goat serum for 15min and permeabilized with Triton X-100 for 5min. The permeabilized cells were incubated with primary antibody (pVIIIa serum or pVIIIb serum, 1:300) in 0.1M PBS for 1hr at room temperature. The pVIII-antibody complex was detected by Cy-2 conjugated goat α-rabbit secondary antibody (Jackson Immunoresearch) and the cells were mounted in medium

Table 3.2.1. List of primers

Primer name	Primer sequence
VIII-F	5’-CATATGAGCAAAGAAATTCCCACAC
VIII-R	5’-GAATTCCGCTATAACCGCTCACAG
XhoI VIII F	5’-ACTCGAGATGAGCAAAGAAATTCCCA
EcorI VIII R	5’-AGAATTCAGCTATAACCGCTCACAGAG
pVIII-D1-F	5’ CCGCTAGCCATATGAGCAAAGAAATTCCC
pVIII-D1-R	5’ GAATTCCTCTGGGATCAATCAG
pVIII-D2-R	5’ CGAATTCCGGTGAGCGGGATAAGGGTTCG
pVIII-D3-F	5’ GCTAGCATGGCCTCCTGCTTAAGACCAGAT
pVIII-D3-R	5’ CGAATTCCGCTATAACCGCTCACAGAGTTTGC
pVIII-D4-F	5’ TAGCTAGCATGCAGTGGGCCGCCCACCTCATCAAAC
pVIII-D5-F	5’ GGCTAGCATGGGACCTGATATGATCCACGACGTT
SnaBI-F	5’ GCGGCCCATCTACGTATTAGTCATCGCTATTAC
EcorI-R	5’ TGAATTCCAAGGATGCGGTTTTGGGCGTCAC
NLS-EY-HindIII-F	5’ GAAGCTTATGACTCAGTCGGCCATTACCGCCACTCC
NLS-EY-XbaI-R	5’ TAGCCCTCTAGAGTCGCGGCCGCTTAC
gbeta-NheI-R	5’ GGCCGCTAGCTCTGGGATCAATCAGATTCCTGGG

containing DAPI (Vectashield) and visualized under Zeiss LSM 5 laser scanning con-focal microscope.

3.2.6. Transfection assay

Monolayers of Vero cells (60-70% confluent) in two well chamber slides were incubated with Opti-MEM I reduced serum media (Gibco) for 2hrs prior to transfection. The cells were transfected with 1µg / well of individual plasmid DNA using Lipofectamine 2000 (Invitrogen). After 36hrs post transfection, the cells were visualized as described above.

3.2.7. Recombinant protein expression and protein purification

Glutathione S-transferase (GST) and GST fusion proteins were purified using GST beads (GE Health care) as per the instructions of the manufacturer. The recombinant proteins: GST-NLS-GFP and GST-RanQ69L (Imamoto et al., 1995), importins- α-1, α-3, α-7 and β (Friedrich et al., 2006, Melen et al., 2003) were expressed in *E.coli* BL21 cells and purified as previously described (Imamoto et al., 1995; Paterson et al., 2012). The purified proteins were dialyzed using Slide-A-Lyzer dialysis cassette (Thermo scientific). The concentrations of the proteins were measured by Bradford assay (Bio Rad) using Ultrospec® 3000 spectrophotometer (Pharmacia Biotech).

3.2.8. GST-pull down assay

Plasmid pRSET.pVIII DNA (0.8µg) was used to synthesize radio-labeled pVIII *in-vitro* by utilizing a TNT T7 Coupled Reticulocyte Lysate System (Promega) in the presence of [^{35}S]-methionine (Perkin Elmer). Prior to use, glutathione sepharose beads were washed three times with GST binding buffer (0.54M NaCl, 2.7mM KCl, 10.15mM Na_2HPO_4, 1.75mM KH_2PO_4,

0.01M $MgCl_2$, 1µg/ml each Aprotinin and Leupeptin, 1% Triton X-100, 1mM PMSF and DNAse I). Fifteen µgs of each GST or GST fusions of importins- α-1, α-3, α-5, α-7, or β were incubated individually with 20µl of glutathione sepharose beads plus 10µl of *in-vitro* synthesized pVIII at + 4^{o}c over night on a nutator. The beads were washed three times, 10min each with PBS. The bead bound proteins were separated by using 10% SDS-PAGE and were fixed in de-stain solution (200ml methanol, 100ml glacial acetic acid and 700ml double distilled H_2O) for 30min and dried onto filter paper at 80^{o}c for 2hrs. Subsequently, the the dried gel was exposed to a phosphor screen (Kodak) overnight and visualized on a Molecular Imager FX (Bio-Rad).

3.2.9. *In-vitro* nuclear import assay

The *in vitro* nuclear import assay was carried out using digitonin (selectively permeabilizes the cell membrane leaving the nuclear membrane intact) permeabilized MDBK cells as described (Adam et al., 1990) with some modifications (Paterson et al., 2012). Briefly, the cells that were incubated with complete transport solution containing GST-NLS-GFP were processed as described (Paterson et al., 2012). The cells incubated with complete transport solution containing GST.pVIII were permeabilized using 0.05% Triton X-100 in PBS for 5min, then stained with anti-pVIII (a or b) serum followed by Cy3 conjugated goat anti-rabbit IgG. Finally, the cells were washed in PBS and mounted on slides in Vectashield mounting medium containing DAPI and visualized under Zeiss LSM 5 laser scanning con-focal microscope.

3.3. RESULTS

3.3.1. Sequence analysis of pVIII protein

To identify conserved regions in pVIII proteins of different adenoviruses we used the computer program BLOSUM 62 application in Clone manager version 9.3. As seen in Figure 3.3.1, sequences at the N-terminus and C-terminus appear conserved in pVIII homologues from fourteen different adenovirus species including BAdV-3. Secondly, BAdV-3 pVIII amino acid sequence was analyzed using different online databases to identify canonical intracellular localization signals. None of them predicted the presence of a classical NLS in BAdV-3 pVIII.

3.3.2. Analysis of expression of pVIII by Western blot

To characterize BAdV-3 pVIII, pVIII protein specific anti-sera pVIIIa and pVIIIb were generated against peptides VIIIa (THVEMPRNEVLEQHLTSHGAQIA) and VIIIb (LNPYSGPPDSYPDQFIRHYNVYSNSVSGYS), respectively. Sera collected after the final protein boost was analyzed by Western blot. As seen in Figure. 3.3.2A, mixture of anti-pVIIIa and anti-pVIIIb sera detected a 24 kDa protein between 12 to 48 hrs post BAdV-3 infection. Interestingly, an additional protein of 8 kDa was detected between 24-48 hrs post infection (Figure. 3.3.2A). No such proteins could be detected in mock infected cells (Figure. 3.3.2A, lane Cx).

3.3.3. Detection of pVIII in mature and immature BAdV-3

To determine the identity of the two proteins and if pVIII is part of the virions, immature and mature virions were purified from BAdV-3 infected MDBK cells by double cesium chloride density gradient centrifugation technique (Figure. 3.3.2B). Proteins from mature virions, empty capsids, and lysates of mock or BAdV-3 infected MDBK cells were analysed by Western blot

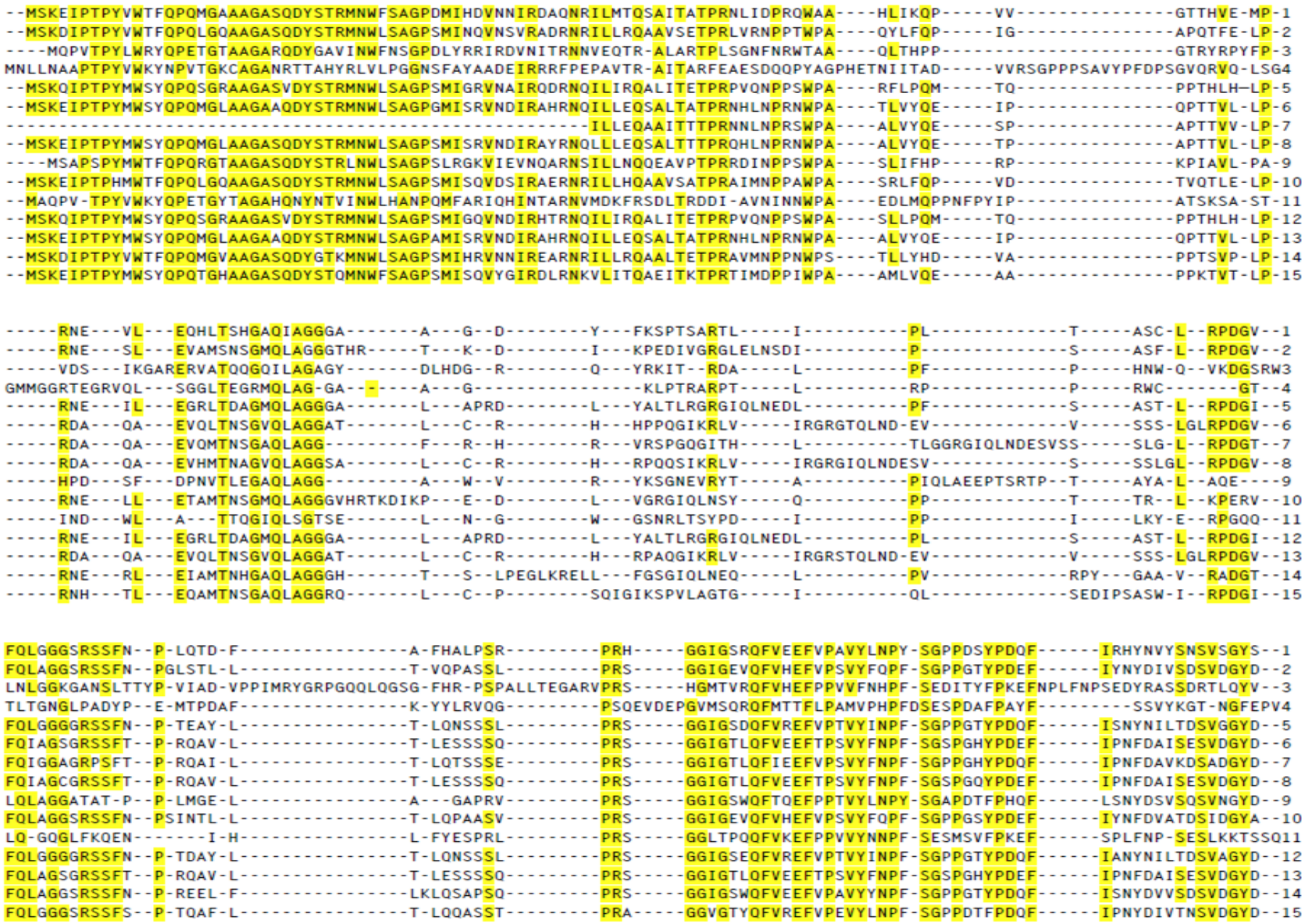


Figure 3.3.1 Areas of amino acid identity between BAdV-3 pVIII and 14 homologues of adenovirus pVIII. The numbers on the right corresponds to the different adenoviruses. The GenBank Accession number of each sequence is also included. ; Number 1 (BAdV-3; AAD09733.1); 2 (BAdV A; , YP_094045.1), 3 (Duck adenovirus 1; CAA68124.1), 4 (Fowl adenovirus 10; AAB88 280.1), 5 (HAdV-2; AAB42039.1), 6 (HAdV-4; AAN64737.1, 7 (HAdV-5; CAA26780.1), 8 (HAdV-7; AP_000554.1), 9 (murine adenovirus 3; YP_002822222.1), 10 (Ovine adenovirus A; AP_000018.1), 11 (Ovine adenovirus D; NP_659528.1), 12 (porcine adenovirus-3BAA76972.1), 13 (Simian adenovirus 25; AP_000319.1), 14 (Tree shrew adenovirus; YP_068076.1) and 15 (Canine adenovirus 2, AAB22306.1)

using anti-pVIIIa sera (panel C) or anti-pVIIIb sera (panel D). As seen in Figure3.3.2C, anti-pVIIIa sera detected a band of 24 kDa in empty capsids (lane b) and BAdV-3 infected cells (lane c). No such protein could be detected in mature virions (lane d) or mock infected MDBK cells ((lane a). Anti-pVIIIb sera detected proteins of 24 kDa and 8 kDa in BAdV-3 infected cells (Figure 3.3.2D, lane c; Fig. 3.3.2H). Only 24 kDa protein is detected in empty capsids (Figure 3.3.2D, lane b; Fig. 3.3.2H). Similarly, only 8 kDa protein is detected in mature BAdV-3 virions (Figure 3.3.2D, lane d; Figure3.3.2H), which corresponds to one of the adenovirus specific protease cleavage product (Figure3.3.2 H). No such protein was detected in immature virions using anti-pVIIIb (Figure 3.3.2D, lane b). In addition, a higher molecular weight protein of ~110 kDa could be detected in BAdV-3 infected cells (Figure 3.3.2D, lane c), purified empty capsids (Figure. 3.3.2D, lane b) or mature BAdV-3 virions (Figure 3.3.2D; lane d). Based on Mass spectrometry analysis, the ~110kDa protein was identified as hexon.

To determine if association between pVIII and hexon is through a disulfide bond, the samples were treated with 40% (Figure 3.3.2E; lane a), 30% (Figure 3.3.2E; lane b), 20% (Figure 3.3.2E lane c) of β-mercaptoethanol before analyzing by Western blot. As seen in Figure 3.3.2E, no significant difference could be detected in the pattern of the expression of cleaved fragment of pVIII or hexon (lanes a-c). To determine if pVIII-Hexon interaction is specific, proteins from mock or BAdV-3 were ananalyzed by Western blot using GST-pVIII absorbed anti-pVIII sera. As seen in Figure 3.3.2F, preabsorption of anti-pVIII with GST-pVIII purified protein abolished the detection of both pVIII and hexon protein in BAdV-3 infected cells (lane b). However, anti-pV serum detected a specific protein of 56 kDa in same BAdV-3 infected cells (lane b). No such protein could be detected in mock infected cells (lane a)

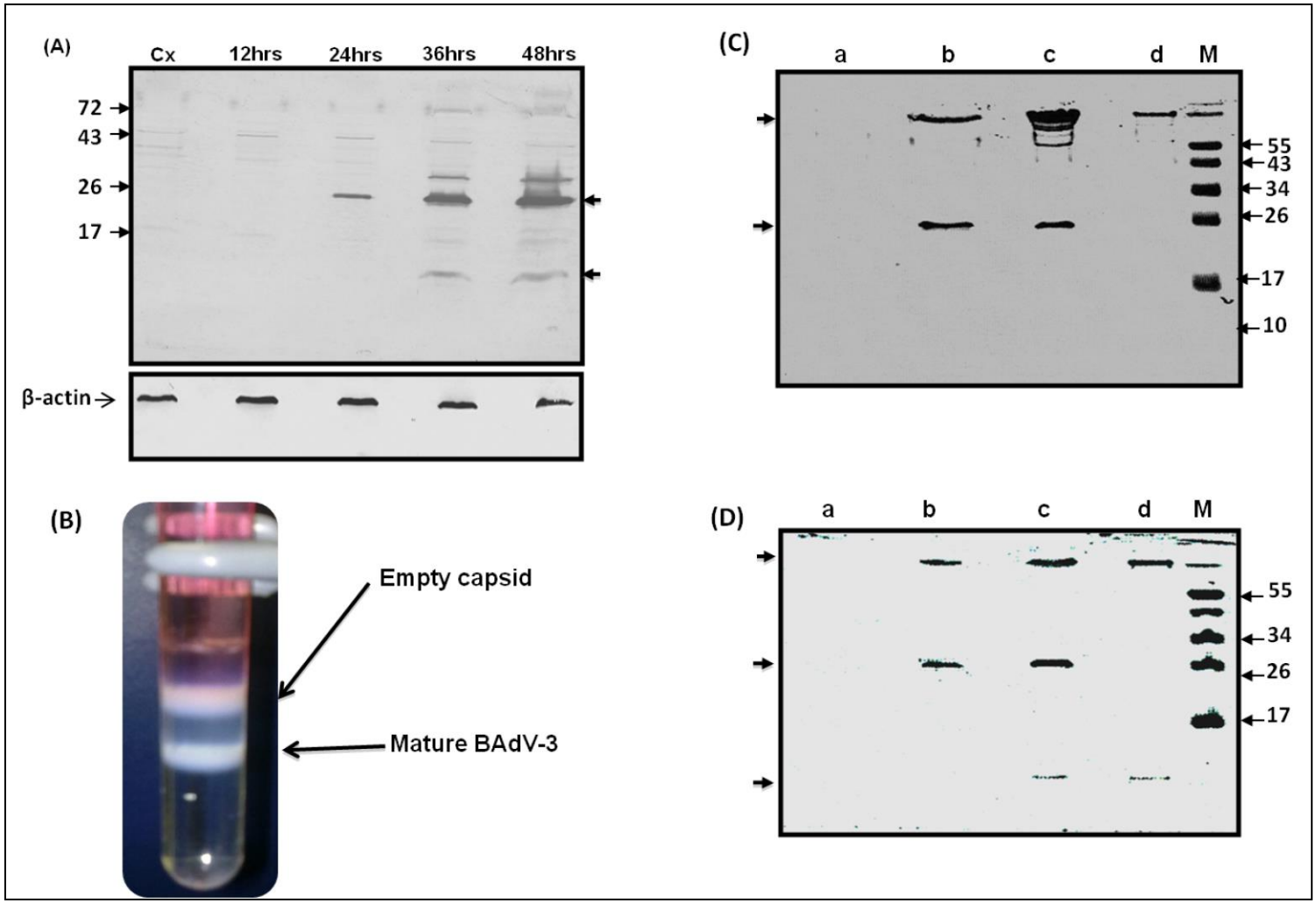
To determine the localization of pVIII in infected cells, Proteins from nuclear or cytoplasmic fractions purified from BAdV-3 infected cells were separated by 15% SDS-PAGE and analyzed by Western blot. As seen in Figure 3.3.2G, anti pVIII detected a 24 kDa protein in both nuclear and cellular fraction of BAdV-3 infected cells. As expected, anti-fibrillarin serum detected fibrillarin protein in purified nuclear fraction but not in purified cytoplasmic fraction of BAdV-3 infected cells.

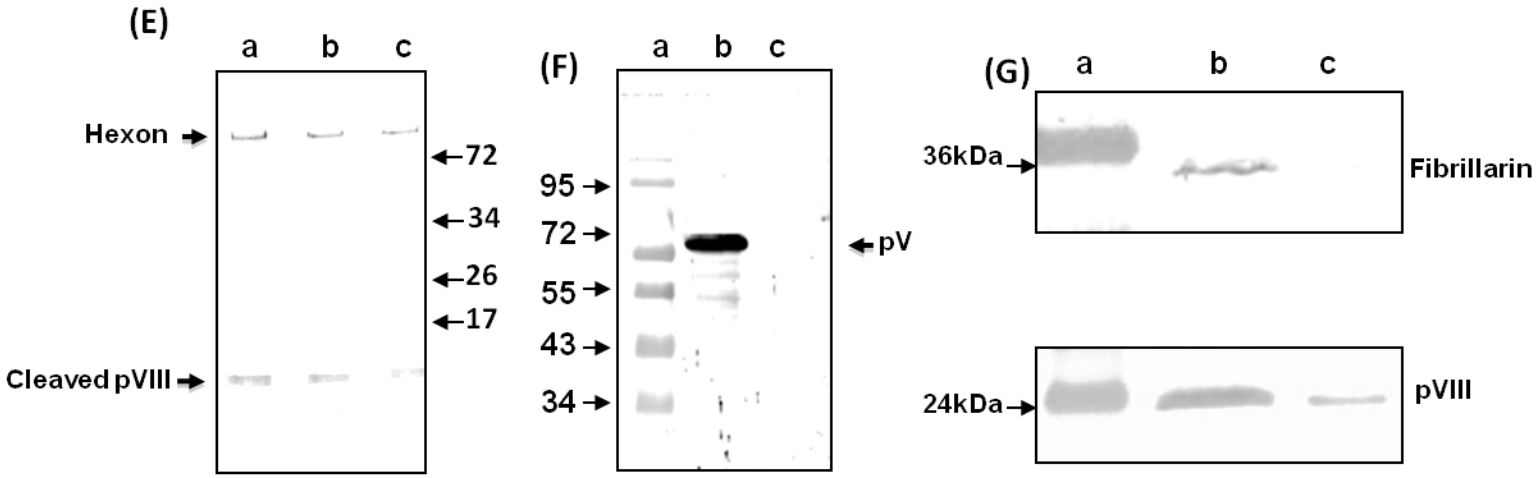
3.3.4. Sub cellular localization of pVIII

To determine the intracellular localization of pVIII, MDBK cells were infected with BAdV-3 and analyzed by immuno-staining using anti-pVIII (a and b) sera. As seen in Figure 3.3.3, pVIII localizes both in the cytoplasm and in the nucleus of BAdV-3 infected cells (panel a). No fluorescence was observed in uninfected MDBK cells (panel b). To examine if nuclear localization of pVIII requires any other protein, Vero cells were transfected with plasmid pEY.pVIII (1µg/well of 2 well chamber slide) DNA and examined under laser scanning con-focal microscope 36 hrs post transfection. As seen in Figure 3.3.3, (panel c), pVIII localizes in the nucleus and in the cytoplasm of transfected cells and appears in the form of dots.

3.3.5. *In-vitro* nuclear import of pVIII

To investigate the biochemical details of nuclear import of pVIII, we performed *in vitro* nuclear import assay with digitonin permeabilized MDBK cells. The plasma membranes of MDBK cells seeded on cover slips were permeabilized with 40µg/ml of digitonin and transport mixes were prepared as indicated in the materials and methods. GST.pVIII was used as a cargo


(A)
Cx
12hrs
24hrs
36hrs
48hrs
72
43
26
17
β-actin
(B)
Empty capsid
Mature BAdV-3
(C)
a
b
c
d
M
55
43
34
26
17
10
(D)
a
b
c
d
M
55
34
26
17


(E)
a
b
c
Hexon
72
34
26
17
Cleaved pVIII
(F)
a
b
c
95
72
55
43
34
pV
(G)
a
b
c
36kDa
Fibrillarin
24kDa
pVIII

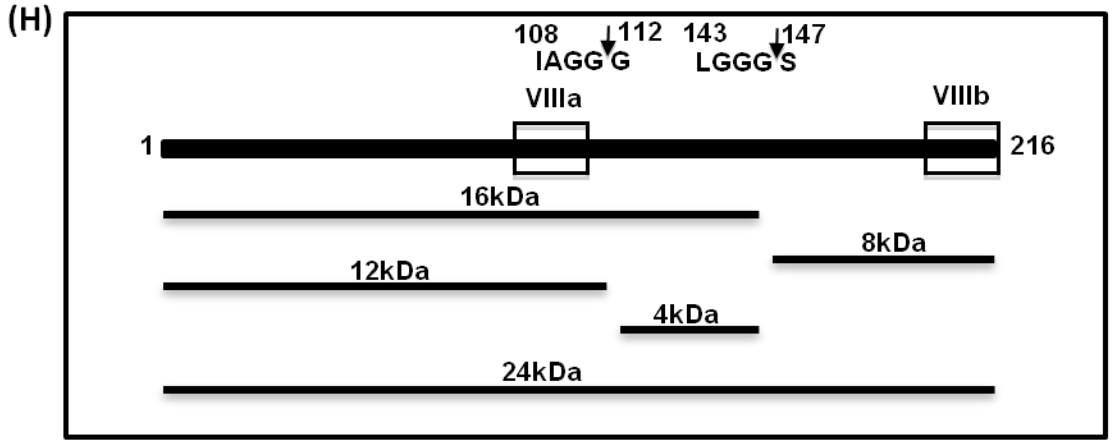

(H)
108
112
143
147
IAGG G
LGGG S
VIIIa
VIIIb
1
216
16kDa
8kDa
12kDa
4kDa
24kDa

Figure 3.3.2 Western blot (A) Proteins from the lysates of MDBK cells infected with wild-type BAdV-3 at an MOI of 5 collected at indicated times post infection were separated by 15% SDS-PAGE gel, transferred to PVDF membrane and probed in Western blot using mixture of anti-pVIIIa and α-pVIIIb antibodies. Uninfected MDBK cell lysates (Cx) were used as a negative control. β-actin was used as a loading control. Sizeof markers in kDa are indicated on the left. The pVIII proteins are indicated by arrows on the right. **(B) CsCl2 purification.** Purified empty capsids and mature virion bands after double Cesium chloride density gradient centrifugation. **(C, D, E & F) Analysis of pVIII of BAdV-3 in mature and immature virus**. **(C)** Proteins from the lysates of mock infected MDBK cells (a), BAdV-3 infected MDBK cells (c), $CsCl_2$ purified empty capsids (b) or mature virions (d) were separated by 15% SDS-PAGE and analyzed by Western blot using anti-pVIIIa, Size marker (M) bands in kDa are indicated on the right. The hexon and pVIII proteins are indicated by arrows on the left **(D)** Proteins from the lysates of mock infected MDBK cells (a), BAdV-3 infected MDBK cells (lane c), $CsCl_2$ purified empty capsids (lane b) or mature BAdV-3 virions (lanes d) were separated by 15% SDS-PAGE and analyzed by Western blot using anti-pVIIIb serum. (E) Samples in lanes a, b and c are mature virions treated with 40, 30 and 20% β-ME, respectively. Size markers in kDa are indicated on the right. The hexon and pVIII proteins are indicated by arrows on the left (F) Proteins from lysates of mock infected (lane c) or BAdV-3 infected (Lane b) infected MDBK cells were separated by 15%SDS-PAGE and analysed by Western blot using anti-pVIII serum pre-absorbed with purified GST-pVIII protein. Anti-serum detecting BAdV-3 pV was used as a control. (G) Proteins from lysates of nuclear or cytoplasmic fractions isolated from BAdV-3 infected cells were separated by 15% SDS-PAGE and probed in Western blot with anti-fibrillarin serum (nuclear marker) or anti-pVIII serum. **(H) BAdV-3 pVIII cleavage fragments.** Schematic

of potential polypeptides after cleavage at predicted protease cleavage sites (shown by arrows) of BAdV-3 pVIII. Expected sizes of cleavage fragments in kDa are depicted. The location of pVIII specific peptides VIIIa (AA 85-109) and VIIIb (AA 187-216) used for producing antisera pVIIIa and pVIIIIb, respectively is depicted.

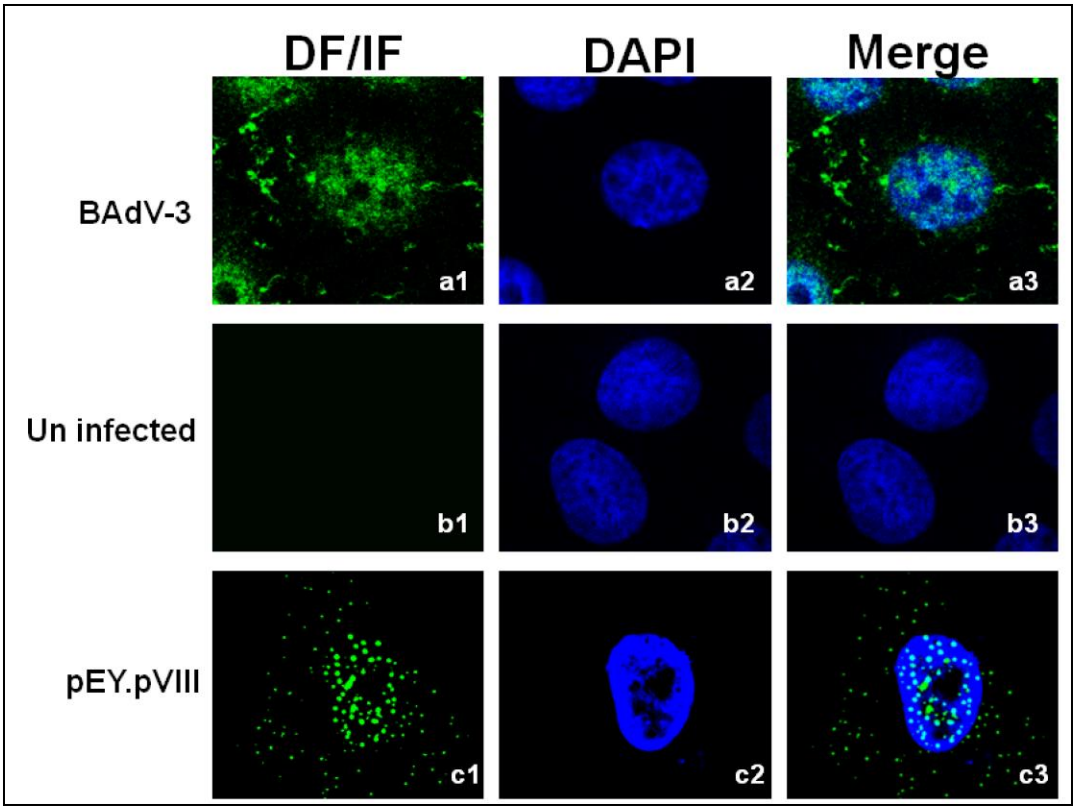


Figure 3.3.3 Sub cellular localization of pVIII. MDBK cells were infected with wild-type BAdV-3 at an MOI of 5 (a1-a3) or mock infected (b1-b3). Vero cells were transfected with 1µg of plasmid pEY.pVIII DNA (panel c1-c3). At 36 hrs post infection or 48 hrs post transfection, the sub cellular localization of pVIII in the cells were visualized by indirect immunofluorescence (a1-a3; b1-b3) using α-pVIIIa & b serum as primary antibody and cy-2 conjugated goat anti rabbit antibody as a secondary antibody or direct fluorescence (c1-c3) under laser scanning LSM 5 Confocal Microscopy. The nuclei were stained with DAPI (a2, b2, c2).

protein and a well characterized GST-NLS-GFP (Adam et al., 1990) was used as a positive control for nuclear transport.

As expected, GST-NLS-GFP, a control cargo containing classical importin- α/ β dependent NLS was efficiently imported into the nucleus in the presence of complete transport mixture (Figure 3.3.4; panel a). No such import could be detected in the absence of reticulocyte lysate (source of cytosolic nuclear transport factors) (Figure 3.3.4 panel b) or ATP regenerating system (Figure 3.3.4; panel c). Similarly, GST-pVIII was efficiently imported into the nucleus in the presence of complete transport mixture (Figure 3.3.4; panel g). No such nuclear import could be detected in the absence of rabbit reticulocyte lysate (panel h) or ATP regenerating system (panel i). To determine if nuclear transport is mediated by a Ran-dependent mechanism, the nuclear import was analyzed in the presence of the GTPase deficient RanQ69L mutant (preloaded with 10mM GTP), As seen in Figure 3.3.4, addition of GTPase deficient RanQ69L protein, which promotes disassembly of nuclear import complexes (Richardson et al., 1988) completely inhibited the nuclear import of GST-NLS-GFP (panel d) and GST-pVIII (panel j). In addition, nuclear import of both GST-NLS-GFP and GST-pVIII proteins was effectively inhibited, when the experiment was performed on ice in the presence of complete transport mix (Figure 3.3.4; panel f, l). Addition of WGA, a lectin that binds glycosylated nucleoporins and inhibits nuclear pore complex function also inhibited nuclear import of GST-NLS-GFP (panel e) and GST-pVIII cargo proteins (panel k).

3.3.6. Identification of nuclear localization domain of pVIII

To identify the domain(s) responsible for nuclear localization of pVIII, a panel of plasmids encoding mutant pVIII proteins fused in frame to EYFP gene was constructed (Figure

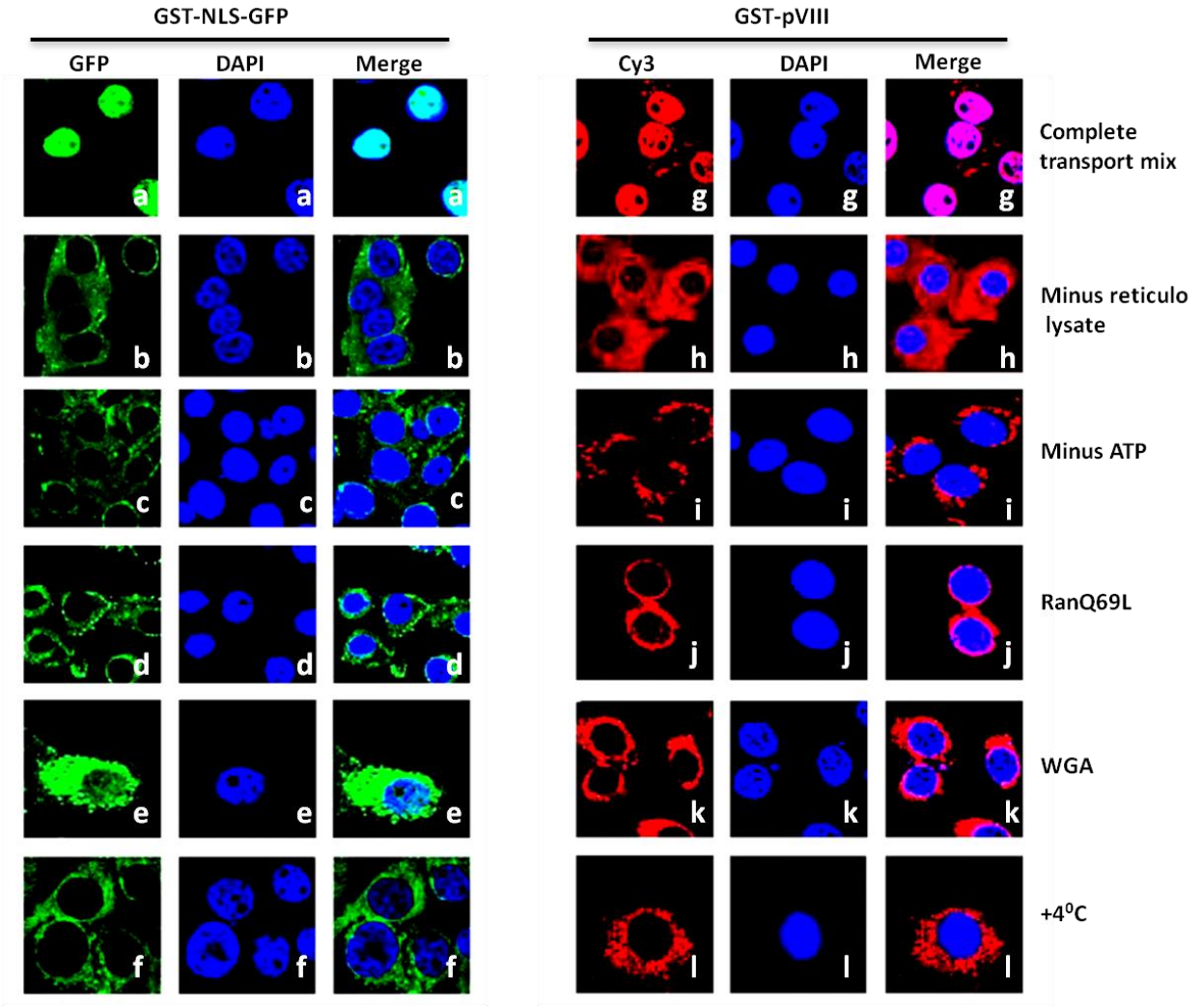


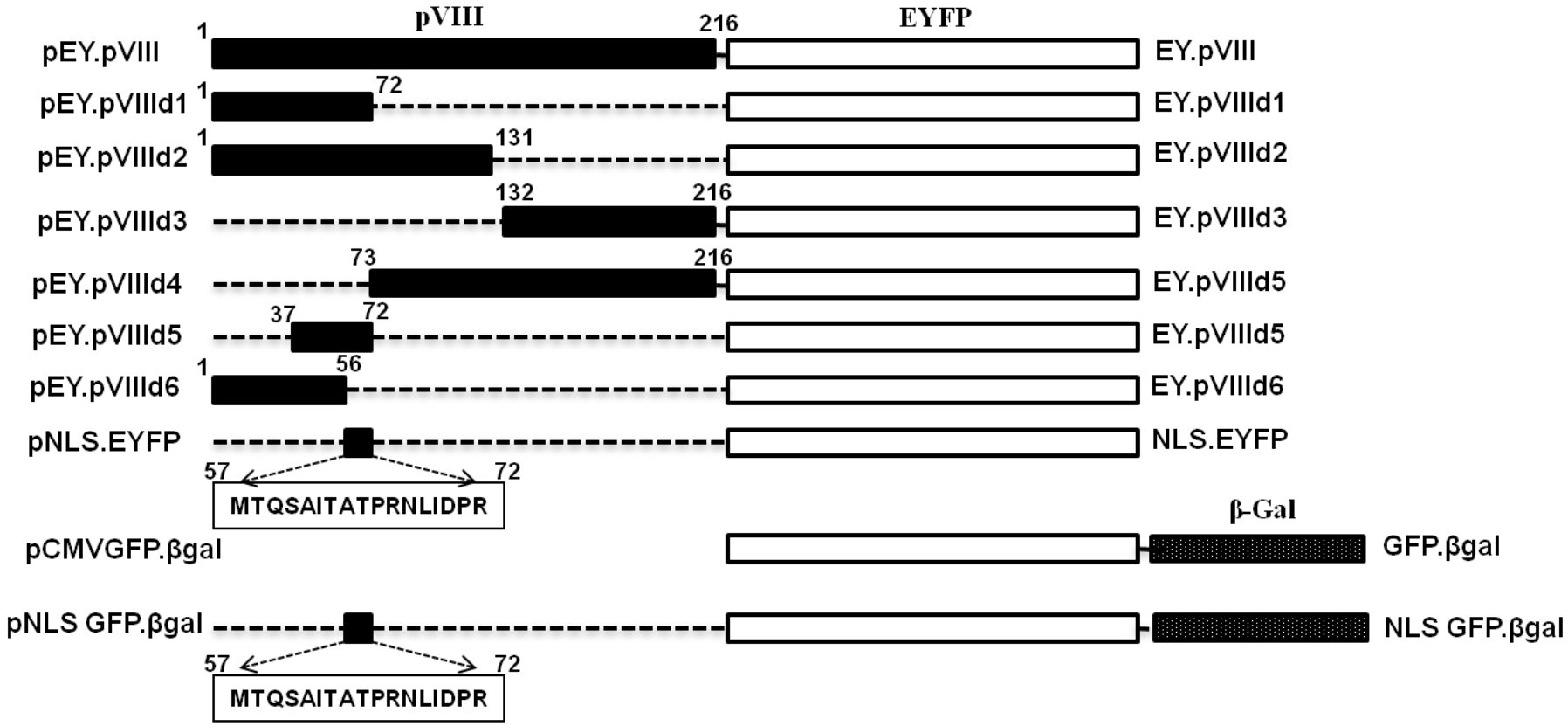
Figure 3.3.4 Nuclear import assay in digitonin permeabilized cells. MDBK cells were permeabilized with 40µg/ml of digitonin and incubated with GST-NLS-GFP (panels a-f) or GST.pVIII (panels g-l) in the presence (panels a, d, g, j) or absence (panels b, c, h, i) of indicated components of the transport mixture. The permeabilized cells were treated with WGA before addition of complete transport mixture (panels e, k) or the experiment with complete transport mix was performed at 4^0C The pictures were taken using Laser scanning LSM 5 Confocal Microscope.

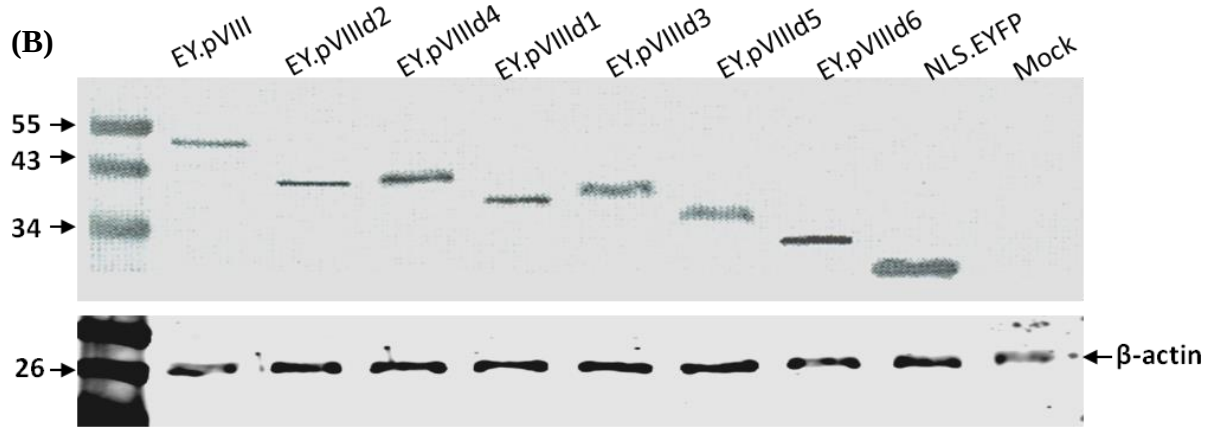
3.3.5A) and their expression analyzed by Western blot using anti GFP antibody (Abcam) (Figure 3.3.5B). Vero cells (~70% confluent) were transfected with individual plasmid DNAs and analyzed by confocal microscopy at 36hrs post transfection. As seen in Figure 3.3.5C, deletion of amino acids 1-131 (pEY.pVIIId3) (panel d) and 1-72 (pEY.pVIIId4) (panel e) resulted in the localization of pVIII in the cytoplasm of the transfected cells. In contrast, deletion of amino acids 73 to 216 (pEY.pVIIId1) (panel b) or 132-216 (pEY.pVIIId2) (panel c) resulted in the localization of pVIII in the nucleus of the transfected cells. Similarly, deletion of amino acids 1-36 and 73-216 (pEY.pVIIId5) (panel f) or 1-55 and 73-216 (pNLS.EYFP) (panel h) yielded nuclear localization of mutant proteins, In contrast, deletion of 57-216 (pVIIId6) (panel g) showed localization exclusively in the cytoplasm. This suggested that potential NLS of pVIII appears to be located between amino acid 56-72.To assess if the identified NLS region of pVIII is able to direct the nuclear import of a hetrologous cytoplasmic GFP-β-gal fusion protein (Wu and Tikoo, 2004), Vero cells were transfected with plasmid pNLS GFP.βgal (amino acids 57-72 of pVIII fused in frame with GFP-β-gal) or plasmid pCMVGFP.βgal DNAs and analysed by confocal microscopy after 36 hrs post transfection. As seen in Figure 3.3.5C amino acids 57-72 of pVIII (panel j) were able to partially direct the heterologous protein into the nucleus, while pCMVGFPβ-gal localized solely in the cytoplasm (panel i).

3.3.7. Interaction of pVIII with importins

Interaction of proteins with the members of importin super family mediate nuclear transport through nuclear pore complexes localized in the nuclear envelop. To determine the involvement and identification of the cellular import receptors, GST-pull down assay was performed, importin

(A)


pVIII
EYFP
pEY.pVIII
1
216
EY.pVIII
pEY.pVIIId1
1
72
EY.pVIIId1
pEY.pVIIId2
1
131
EY.pVIIId2
pEY.pVIIId3
132
216
EY.pVIIId3
pEY.pVIIId4
73
216
EY.pVIIId5
pEY.pVIIId5
37
72
EY.pVIIId5
pEY.pVIIId6
1
56
EY.pVIIId6
pNLS.EYFP
NLS.EYFP
57
72
MTQSAITATPRNLIDPR
β-Gal
pCMVGFP.βgal
GFP.βgal
pNLS GFP.βgal
NLS GFP.βgal
57
72
MTQSAITATPRNLIDPR


(B)
EY.pVIII
EY.pVIIId2
EY.pVIIId4
EY.pVIIId1
EY.pVIIId3
EY.pVIIId5
EY.pVIIId6
NLS.EYFP
Mock
55
43
34
26
β-actin

(C)

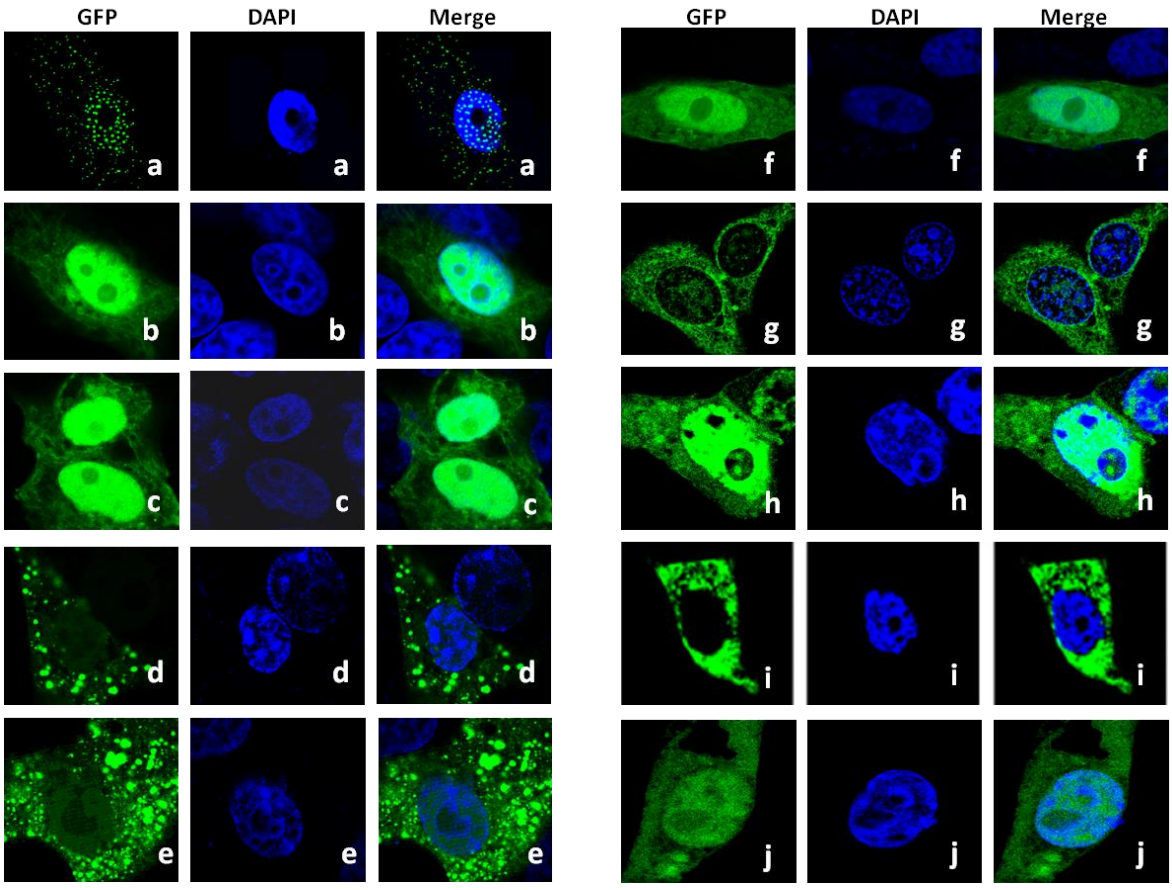

GFP
DAPI
Merge
a
b
c
d
e
f
g
h
i
j

Figure 3.3.5 (A) Schematic representation of BAdV-3 pVIII. The number above the domain represents amino acid number in pVIII. Thick box represents BAdV-3DNA, hollow box represent EYFP\GFP DNA. Thick box with white dots represent beta-Gal DNA. Dotted lines represent deleted regions. The name of the plasmid is on the left. The name of the protein is on the right. **(B) Western blot.** 293T cells seeded on 6well plates were transfected with 2µg of each indicated DNA. 36hr post transfection the cells were lysed with RIPA buffer and run on 12% SDS-PAGE gel and Western blot was performed using anti GFP serum (Abcam), followed by goat anti rabbit IRDye680. Anti actin antibody was used as a loading control. **(C) Confocal microscopy**. About 70% confluent Vero cells were transfected with 1µg of each plasmid: pEY.pVIII (panel a), pEY.VIIId1 (panel b), pEY.pVIIId2 (panel c), pEY.VIIId3 (panel d), pEY.VIIId4 (lane e), pEY.VIIId5 (lane f), pEY.pVIIId6 (lane g), pNLS.EYFP (lane h), pCMV GFP.βgal (i) or pNLSGFP.βgal (J) in two well chamber slides. The intracellular localization of the expressed proteins was examined under Laser scanning LSM 5 Confocal Microscope.

- α-1, α-3, α-5, α-7 and β1 were expressed as GST fusion proteins in *E. coli* (BL21) and purified (Figure 3.3.6A). Purified GST alone or GST fusion proteins coupled to glutathione-Sepharose beads were individually incubated with radiolabelled pVIII synthesized *in-vitro* in the presence of [^{35}S]-methionine using plasmid pRSET. pVIII DNA, pcEY.pVIIId1, pcEY.pVIIId4, pNLS.EYFP or pEYFPN1 and TNT T7 Coupled Reticulocyte Lysate System. The protein bound to beads was separated by 10 or 15% SDS-PAGE and visualized by autoradiography. As seen in Figure 3.3.6B, pVIII interacts with importins- α-1 (lane 3), α-3 (lane 2) and α-7 (lane 4). However, pVIII appears to interact preferentially with importin- α-3. No such binding was observed when purified GST alone (lane 6), GST-importin- α-5 (lane 5) or GST-importin β1 (lane 1) bound to GS beads were used to pull down pVIII. Radiolabelled IVTT pVIII was used as input control (lane 7).

Like pVIII (Figure 3.3.6C, lane 2), GST-importin α-3 interacts with radiolabelled EY.pVIIId1 containing deletion of amino acid 73-216 (Figure 3.3.6C, lane 3). No interaction was observed between GST-importin α-3 and EY.pVIIId 4 containing deletion of amino acid 1-72 (Figure 3.3.6C, lane 5). Finally, like pVIII (Figure 3.3.6D, lane 4) or EY.pVIIId1 (Figure 3.3.6D, lane 5), GST-importin- α-3 bound to GST beads was able to bind to NLS.EYFP (EYFP fused to amino acid 57-72) (Figure 3.3.6D, lane 2). No interaction was observed when GST-importin- α3 was used to pull down EYFP (Figure 3.3.6, lane 3) or GST alone was used to pull down NLS.EYFP (Figure 3.3.6D lane 6).

3.3.8. Nuclear import of pVIII in the presence of inhibitory peptides

To further confirm the involvement of importins in the nuclear import of pVIII, we performed nuclear import blocking assays using specific peptides that can selectively block the

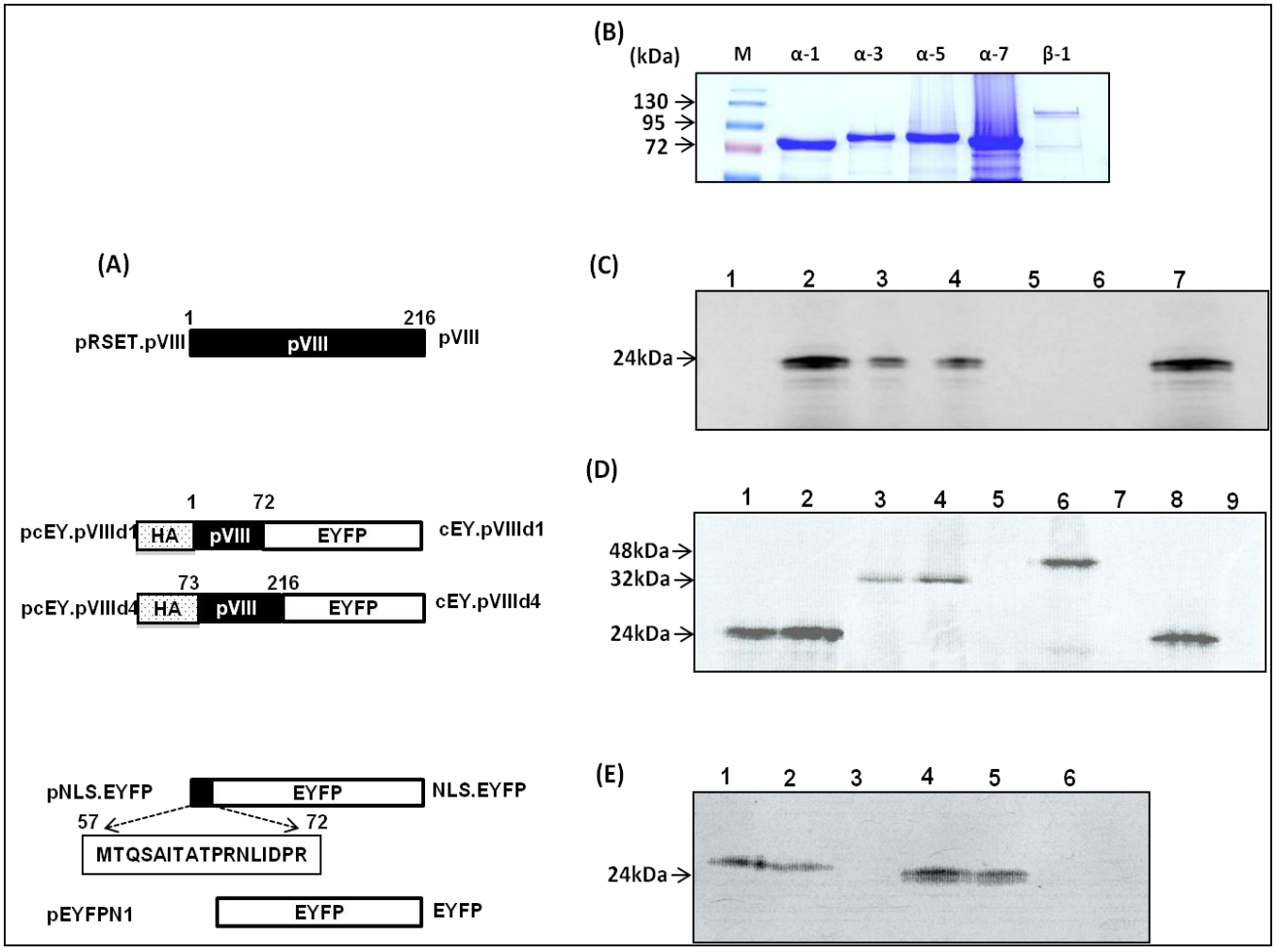

(A)
1
216
pRSET.pVIII
pVIII
pVIII
1
72
pcEY.pVIIId1
HA
pVIII
EYFP
cEY.pVIIId1
73
216
pcEY.pVIIId4
HA
pVIII
EYFP
cEY.pVIIId4
pNLS.EYFP
EYFP
NLS.EYFP
57
72
MTQSAITATPRNLIDPR
pEYFPN1
EYFP
EYFP
(B)
(kDa)
M
α-1
α-3
α-5
α-7
β-1
130
95
72
(C)
1
2
3
4
5
6
7
24kDa
(D)
1
2
3
4
5
6
7
8
9
48kDa
32kDa
24kDa
(E)
1
2
3
4
5
6
24kDa

Figure 3.3.6 *In-vitro* interaction of pVIII with transport receptors. **(A). Schematic diagram of BAdV-3 pVIII**. The solid box represents BAdV-3 pVIII DNA, open box represent EYFP DNA. Open box with white dots represent HA tag. The amino acid numbers of pVIII protein are depicted. Plasmid names are on the left and protein names are on the right **(B) Coomassie brilliant blue staining of purified transport receptors. (C-E) GST pull down assay. (C)** *In-vitro* transcribed and translated (IVTT) radio labeled pVIII (lane 7) was incubated with each importins- α-1 (lane 3), α-3 (lane 2), α-7 (lane 4), α-5 (lane 5), β-1 (lane 1) or GST (lane 6). **(D)** IVTT radiolabelled individual wild-type pVIII (lane 2), cEY.pVIIId1 (Lane 4), EYFP (lane 8) or cEY.pVIIId4 (lane 6) was incubated with importin- α-3 (lanes 1, 3, 5 and 7, respectively) or IVTT radiolabelled pVIII with GST (lane 9). **(E)** IVTT radiolabelled NLS.EYFP (lane 1) or wild-type pVIII (lane 4) was incubated with importin- α-3 (lanes 2 and 5, respectively) or IVTT radio labelled NLS EYFP was incubated with GST (lane 6) or IVTT radiolabelled EYFP with importin- α-3 (lane 3), respectively. The incubation was performed overnight at $+4^0$c on a nutator and pulled down with glutathione sepharose beads. The proteins were separated by 10% SDS-PAGE. Subsequently, the gel was exposed to phosphor screen overnight and visualized on a Molecular Imager FX using Quantity One software.

activities of importin- α/β (Gorlich et al., 1996a,b) or transportin 3 (Lai et al., 2001). Specific peptides representing 41 amino acid importin- β binding domain of importin α [IBB importin- α (Gorlich et al., 1996a)], importin- α binding domain of Ycbp80 protein (Gorlich et al., 1996b; Welch et al., 1999) and a 29 amino acid peptide containing eight RS repeats flanked by two arginine rich stretches ([SR1], Lai et al., 2001) were synthesized and added individually to the transport mix with 1mM concentration (50-fold molar excess). As expected (Figure 3.3.7A), addition of Ycbp80 peptide (panel b) or IBB domain peptide (panel c) to the transport mixture blocked the import of GST-NLS-GFP to the nucleus. Similarly, (Figure 3.3.7B) addition of Ycbp80 peptide (panel b) or IBB domain peptide (panel c) to the transport mixture blocked the import of GST-pVIII. The transport of either GST-NLS-GFP (Figure 3.3.7A) or GST-pVIII (Figure 3.3.7B) was not affected in the presence (panel d) or absence (panel a) of SR1 peptide (binds to transportin-SR1) to transport mixture.

3.4. DISCUSSION

Adenovirus protein pVIII is the least characterized core protein. It is present as 120 copies/virion (Reddy et al., 2010) and appears to be involved in linking the core of the virus with the inner surface of the adenovirus capsid (Rohn et al., 1997) thus playing a role in the stability of virion structure (Liu et al., 1985). Recent study suggests that porcine adenovirus-3 pVIII interact with IVa2, which may suggest its role in genome packaging (Singh et al., 2005). Moreover, cleavage of core proteins including pVIII by adenovirus protease appears essential for maturation of progeny virions (Gastaldelli et al., 2008). Although pVIII synthesized as precursor protein is present in empty capsids, only the cleaved form of pVIII protein is present in mature

adenovirus virion (Chelius et al., 2002; Takahashi et al., 2006). The present study was performed to

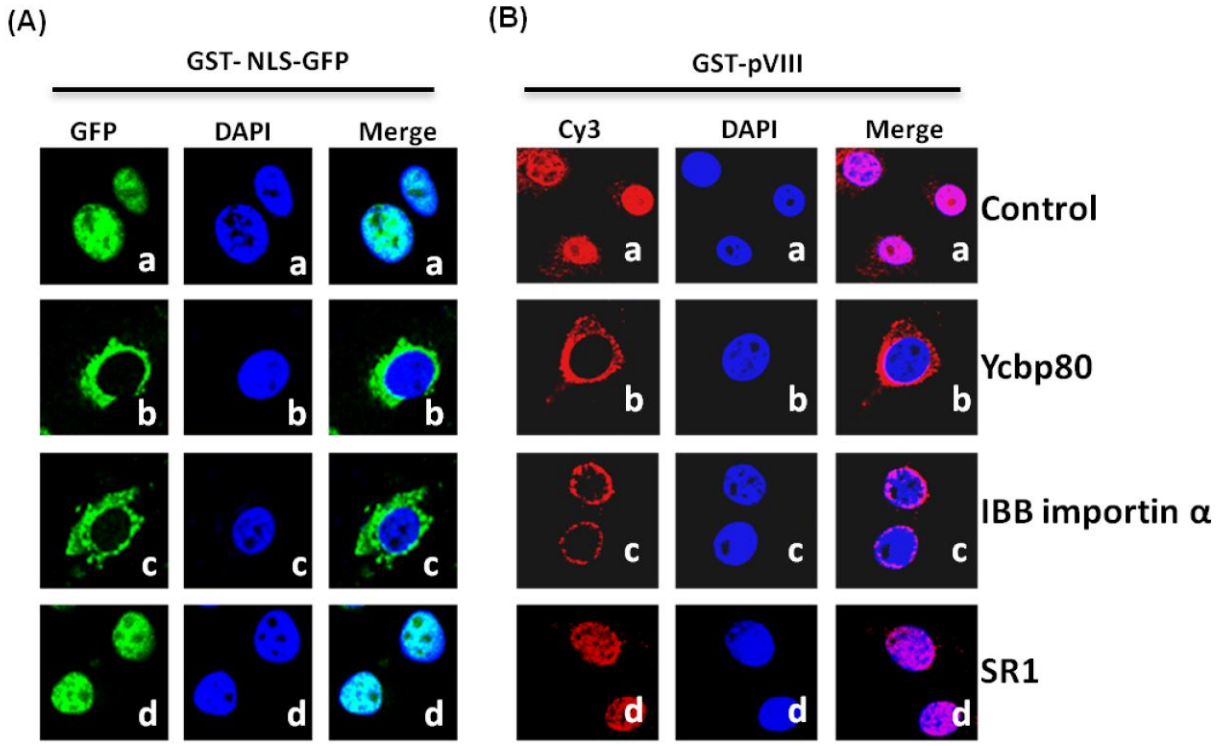


Figure 3.3.7 *In-vitro* nuclear import blocking of pVIII with specific peptides.

Digitonin permeabilized MDBK cells grown on cover slips were incubated with complete transport mix containing either IBBrpl23a (panel b), IBB importin-α (panel c), SR1 peptide (panel d) or complete transport mix without inhibitory peptides (panel a). The cells were analyzed by Laser scanning LSM 5 Confocal Microscopy.

characterize and determine the functional domains of bovine adenovirus-3 pVIII.

The pVIII mRNA is predicted to encode protein of 216 amino acids, which is expressed as a 24kD precursor pVIII protein detected first between 12-24 hrs post infection. Like HAdV-5 (Vellekamp et al., 2001), the pVIII is also detected in the immature but not in mature BAdV-3 capsids. In contrast, 8kDa cleaved pVIII could be detected in mature BAdV-3 capsids. This suggests that BAdV-3 protease cleaves pVIII at potential cleavage site ^{143}LGGG↓ S^{147} generating the C- terminal 70 amino acid polypeptide, which is part of the mature BAdV-3 virion. By using mass spectrometric analysis purified virions, the N-terminal 112 residues (Liu et al., 2003), C-terminal 70 residues (Takahashi et al., 2006) or both N-terminal (112 residues) and C-terminal (70 residues) fragments of pVIII were detected in HAdV-5 virions (Blanche et al., 2001; Chelius et al., 2002). However, recent CryoEM studies suggest that both N-terminus and C-terminus fragments are present in mature virions (Liu et al., 2010). We detected only the C-terminal fragment in BAdV-3 virions. It is possible that our inability to detect N-terminal pVIII fragment in BAdV-3 virions could be due to less sensitivity of Western blot assay. Alternatively, it is possible that only C-terminal 8 kDa protein of pVIII is present in mature BAdV-3 virions. Support for the latter comes from the fact that anti-pVIIIb sera raised against peptide (amino acid 187-216) could detect C-terminal (amino acid 147-216) 8kDa cleavage fragment (Figure 3.3.2D, lane d; Figure 3.3.2E, lanes a-c) in purified virions. In contrast, anti-pVIIIa serum raised against peptide (amino acid 85-109) could detect full length pVIII in empty capsids and BAdV-3 infected cells, suggesting its binding affinity was sufficient to detect pVIII in virions. However, no potential N-terminal (amino acid 1-112) cleavage fragment in mature virions (Figure 3.3.2C, lane d).

High-resolution structural studies suggested that pVIII interacts with hexon and IIIa protein in capsid of adenovirus (Martin et al., 2012). Both the precursor and cleaved form of BAdV-3 pVIII appear to be tightly associated with110 kDa protein hexon protein, and binding does not appear to be due to the formation of disulphide bond. Similar association has been observed between hexon and pVIII of egg drop syndrome virion (EDS) (Rohn et al., 1997), which may suggest that pVIII stabilizes the virion during and after biogenesis of the viral particles (Liu et al., 1985).

pVIII dominantly localizes to the nucleus and appears in the form of multiple punctuate dots in transfected and infected cells. This distinct pattern of pVIII in the nucleus suggests the presence of the protein in viral DNA replication compartments and its potential involvement in BAdV-3 replication. A similar pattern of expression has been indicated for ICP4, ICP8 and VP5 proteins of herpes simplex virus-1 (de Bruyn et al., 1998) and BZLF1 proteins of Epstein - Barr virus (Daikoku et al., 2005), which are essential for viral DNA replication.

Several proteins transported to the nucleus possess classical NLSs containing one or two clusters of basic amino acids. However, recent reports have identified non classical NLSs in proteins which help them to locate to the nucleus (Christophe et al., 2000). Interestingly, analysis of BAdV-3 pVIII protein sequence did not identify the presence of classical NLSs. Since proteins less than ~ 40 kDa can passively diffuse into the nucleus through nuclear pore complexes (Lim et al., 2008), it is possible that detection of 24 kDa BAdV-3 pVIII in nucleus could be due to passive diffusion. However, pVIII fused to a cytoplasmic protein EYFP, a protein too large to diffuse through the nuclear pore, is predominantly localized in the nucleus of transfected cells. This suggested that pVIII may contain a non-classical NLS and is actively transported to the nucleus. Several observations support this conclusion. First, deletion mutant

analysis of intracellular localization ofBAdV-3 pVIII proteins (fused in-frame with EYFP) by confocal microscopy suggested that amino acid 57-72 contain a potential NLS. Secondly, migration of pVIII to nucleus is inhibited at 4^{o}C, in the absence of ATP. Thirdly, the nuclear import of pVIII is inhibited in the presence of WGA [which blocks active transport, but not the free diffusion of macromolecules through the NPC (Yonenda et al., 1987)].

Active transport to the nucleus involves binding of protein NLS to the transportin and imporin-α/β heterodimer in the cytoplasm (Gorlich et al., 1995; Imamoto et al., 1995). This NLS recognition complex then docks to the nuclear pore complex (Newmeyer & Forbes, 1988; Richardson et al., 1988) and is subsequently translocated through the pore by an energy dependent and Ran dependent mechanism (Newmeyer et al., 1988, Richardson et al., 1988, Yoneda et al., 1987). The inability of BAdV-3 pVIII to enter into the nucleus in the absence of reticulocyte lysate indicates the involvement of cytosolic nuclear import receptors for its transport. Interestingly, SR1 peptide, which inhibits transportin- 3 mediated nuclear import did not affect the nuclear localization of pVIII. In contrast, IBB importin peptide (inhibits binding of importin- α to importin- β) and Ycbp80 peptide (which competes for binding to importin- α) inhibits the nuclear transport of pVIII. These results suggest that nuclear transport of BAdV-3 pVIII is mediated by imporin-α/β pathway.

In addition, the transport of BAdV-3 pVIII from the cytoplasm to the nucleoplasm is Ran dependent. Transport receptors use RanGTP binding as a means to regulate their interactions with cargoes or adaptor molecules (Jakel & Gorlich, 1998). The Ran GTP gradient across the nuclear envelope has been proposed to control transport receptor-substrate interactions in a compartment-specific manner (Gorlich et al., 1996b).

The presence of different isoforms of importin-α may imply that each isoform imports specific substrates into the nucleus (Goodwin & Whitehouse, 2001). However, it is well described that all importin-α isoforms can import most cargo proteins with a comparable efficiency (Kohler et al., 1999). pVIII binds *in-vitro* with importins-α-1, α-3 and α-7 signifying the effective manipulation of more than one receptor for passage into the nucleus. Similar results have been reported for the nucleoprotein (NP) of influenza virus (Wang et al., 1997), EBNA-1 protein of Epstein Barr virus (Kieff, 1996) and ORF57 protein of γ-2 herpesvirus (Goodwin & Whitehouse, 2001), which bind importin-α-1 and α-5.

In conclusion, our data demonstrates that pVIII is a nuclear protein associated with hexon that is expressed late in infection. It requires nuclear import factors and a non-conventional nuclear localization signal(s) for localization to the nucleus. Moreover, while the N-terminus contains domains involved in interactions with importins and nuclear localization, the C -terminus 70 amino acids appears to be incorporated in mature virion.

4. TRANSITION FROM SECTION-3 TO SECTION-5

In Section-3 the molecular properties of pVIII including its intracellular dynamics have been characterized. To further understand the significance of pVIII in virus host interaction, I investigated the interaction of BAdV-3 pVIII with cellular proteins and determined the biological relevance of the interactions.

5. INTERACTION OF DDX-3 AND BOVINE ADENOVIRUS-3 pVIII MODULATE CELLULAR mRNA TRANSLATION BY EXCLUDING eIFs FROM THE CAP BINDING COMPLEX

5.1. INTRODUCTION

DDX3, a member of DEAD (Asp-Glu-Ala-Asp) box family RNA helicases (Ditton et al., 2004; Gross et al., 2007; Tarn and Chang, 2009), is a 73kDa nucleo-cytoplamic shuttling protein and is biologically active both in the nucleus and the cytoplasm (Lee et al., 2008; Lai et al., 2008). DDX3 is involved in transcription, pre-mRNA splicing, mRNA export, translation, mRNA turn over (Rocak and Linder, 2007; Kwong et al., 2005) and regulation of innate immunity (Oshiumi et al., 2010). Even though there are conflicting reports about the role of DDX3 in cap-dependent translation, evidence over the years support the role of DDX3 in promoting translation initiation (Lee et al., 2008; Lai et al., 2008; Geissler et al., 2012;Shih et al., 2012; Lai et al., 2010). More recently, DDX3 is suggested to be involved in translation initiation of a subset of mRNAs that carry long 5' UTR (Lai et al., 2008) or RNA stem loop at their 5' UTR (Lai et al., 2008; Soto-Rifo et al., 2012). DDX3 may modulate of cellular mRNA translation by interacting with specific eukaryotic translation initiation factors like eIF4E and PABP (Shih et al., 2012; Shih et al., 2008), eIF2α (Lai et al., 2008), and eIF3 (Lee et al., 2008). Similarly, there is strong evidence for the involvement of Ded1, the yeast homologue of DDX3, in translation initiation (Berthelot et al., 2004; Marsden et al., 2006; Chuang et al., 1997; De La Cruz et al., 1997). More recently, ATP dependent activation of translation initiation and interactions with the components of the translation machinery was established for Ded1.

However, interestingly, translation of stalled mRNAs *in-vitro* is repressed by Ded1 (Hilliker et al., 2011).

Analysis of interaction of viral proteins with cellular proteins has not only helped in defining the role of cellular proteins in virus replication cycle but also their role in cellular processes. For instance, interaction of vaccinia virus K7 protein with DDX3 revealed the role of DDX3 in TBK1/IKKε mediated IRF activation (Schroder et al., 2008). The interaction of viral proteins with cellular proteins is of vital importance in the regulation of virus replication, growth and survival. One of these processes involves the translation of mRNAs. Different viruses utilize various strategies to inhibit translation of capped cellular mRNA to facilitate their life cycle. For example; Rubella virus capsid protein inhibits protein translation by sequestering PABP (Ilkow et al., 2008), Picornaviruses like FMD virus inhibit cap-dependent protein synthesis by cleaving, eIF4A and eIF4G (Belsham et al., 2000) and viral RNA translation is retained through the use of an internal ribosome entry sequence whose translation is not inhibited by the cleavage Correspondingly, calciviruses inhibit host cell cap dependent translation by cleaving eIF4GI and eIF4GII (Willcocks et al., 2004). Likewise, adenoviruses facilitates their replication by altering cellular architecture and host cell gene expression (Burgert et al., 2002; Hodge and Scharff, 1969; Martinez-Palomo and Granboulan, 1967; Martinez-Palomo et al., 1967; Philips and Raskas, 1972; Puvion-Dutilleul et al., 1994; Zhai et al., 1994) using mechanisms that include inhibition of transport of cellular mRNAs (Beltz and Flint, 1979) and inhibition of translation of cellular capped mRNAs (Dolph et al., 1988; Huang and Schneider, 1991). Although someof the cellular processes affected by adenoviruses are understood, little is known about the identity of the proteins (viral or cellular) and the mechanisms involved (Russell, 2000).

Recently interest in DDX3 has been stimulated on account of its significance in the development of cancer (Chao et al., 2006; Botlagunta et al., 2008; Chang et al., 2006; Bol et al., 2013) and the life cycle of important pathogens, including vaccinia virus (Schroder, 2010), hepatitis B virus (Wang and Ryu, 2010; Yu et al., 2010), hepatitis C virus (Ariumi et al., 2007; Oshiumi et al., 2010, Owsianka and Patel, 1999; Mamiya and Worman, 1999; You et al., 1999) and human immunodeficiency virus (HIV)-1 (Liu et al., 2011; Yedavalli et al., 2004). Moreover, interaction of DDX3 with a viral protein has defined the new role of DDX3 in the induction of innate immunity (Schroder et al., 2008). Here, we provide evidence suggesting a role for DDX3 in capped mRNA translation and demonstrate that the interaction of a bovine adenovirus-3 late protein pVIII with DDX3 inhibits cellular capped mRNA translation by depleting DDX3 and associated eIFs (eIF3, PABP and eIF4E) from the cap binding complex while not affecting the stability of cellular mRNAs.

5.2. MATERIALS AND METHODS

5.2.1. Cell lines and viruses

African green monkey kidney (Vero) cells were grown in Dulbecco's Modified Eagle's (DMEM; Sigma Aldrich) medium containing 10% heat inactivated fetal bovine serum (FBS) (Sera Care Life Sciences, Inc.). Madin Darby Bovine Kidney (MDBK) cells were grown in minimum essential medium (MEM; Sigma Aldrich) containing 5% FBS. Wild type bovine adenovirus (BAdV)-3 was used to infect MDBK cells at an MOI of 5.

5.2.2. Antibodies

Anti-pVIII recognizes a protein of 24kDa in BAdV-3 infected cells (Ayalew et al., 2014). Anti-DBP recognizes a protein of 48 kDa in BAdV-3 infected cells (Zhou et al., 2001). Anti-DDX3, anti-eIF4G, anti eIF4E, anti eIF3, anti PABP and fluorescence conjugated goat anti-mouse IgG-FITC (Santa Cruz Biotechnology, Inc, USA), Cy-3 conjugated goat anti- rabbit antibody (Jackson Immuno Research), Alexa Flour 680 goat anti-rabbit IgG antibody (Molecular Probes) or IRDye 800 conjugated goat anti-mouse IgG (Li-COR biosciences, Cat # 926-32210) and anti-HA (Sigma Aldrich, Cat # H9658) and anti β-actin MAb (Sigma Aldrich, Cat # A5441) were purchased.

5.2.3. Plasmid construction

Plasmid pcDNA3-RLuc-POLIRES-FLuc (Poulin et al., 1998), containing the poliovirus IRES was a gift from Dr. Nahum Sonenberg, McGill University, Montreal, Canada. Plasmid pcDDX-3 was a gift from Dr. Arvind H. Patel, MRC-University of Glasgow, Center for Virus Research, University of Glasgow, UK. and plasmid pcDNA3.1(neo)3HA DNA was a gift from Dr. Joyce Wilson, University of Saskatchewan. The construction of plasmid pGEX-100K expressing GST.100K fusion protein has been described (Kulshreshtha and Tikoo, 2008). Plasmid GC.linker and plasmid GN.linker were a gift from Dr. Abraham Loyter, Hebrew University, Jerusalem.

a) *pcDNA.3HA*. The cDNA coding for 3HA epitopes was amplified by PCR by using primers 3HA-F and 3HA-R (Table 5.2.1), and plasmid pcDNA3.1 (neo)3HA DNA as a template. The PCR product was digested with *Hind*III-*Eco*RI and ligated to *Hind*III-*Eco*RI digested plasmid pcDNA3.1 (-) (Invitrogen) creating plasmid pcDNA.3HA.

b) *pHA.DX3*. A 2035bp *Eco*RI-*Bam*HI fragment was isolated from plasmid pcDDX-3 and ligated to *Eco*RI -*Bam*HI digested plasmid pcDNA.3HA creating plasmid pHA.DX3.

c) *pEY.VIII.* A 660bp DNA fragment containing pVIII gene was amplified by PCR using primers VIII-F and VIII-R (Table 5.2.1), and plasmid pFBAV302 (Zakhartchouk et al., 1998) DNA as a template. The PCR product was digested with *Eco*RI and ligated to *Afe*I (blunt end repaired with T4 polymerase) - *Eco*RI digested plasmid pEYFPN1 (Clontech) creating plasmid pEY.VIII.

d) Bovine *DDX3 yeast expression plasmids*. A 1938bp DNA fragment was amplified by PCR using primers DDX3-F and DDX3-R (Table 5.2.1), cDNA (synthesized from RNA isolated from MDBK cells) as a template, digested with *Eco*RI-*Bam*HI and ligated to *Eco*RI-*Bam*HI digested plasmid pGBKT7 or pGADT7 creating plasmid pGB.DX3 and pGA.DX3, respectively.

e) *pVIII yeast expression plasmids*. A 660bp DNA fragment containing pVIII gene was amplified by PCR using primers VIII-F and VIII-R (Table 5.2.1), and plasmid pFBAV302 (Zakhartchouk et al., 1998) DNA as a template. The PCR product was digested with *Eco*RI and ligated to *Nde*I (blunt end repaired with T4 polymerase)-*Eco*RI digested plasmids pGBKT7 or pGADT7 creating plasmids pGB.pVIII and pGA.pVIII, respectively.

f) *pGST.pVIII.* A 666bp *Hind* III (blunt end repaired with T4 polymerase) - *Xho*I fragment of plasmid pR.pVIII was ligated to *Aat*II (blunt end repaired with T4 polymerase) - *Xho*I digested plasmid pGEX-5X-1 (GE Healthcare) creating plasmid pGST.pVIII.

i) *pGST.DDX3*. A 2062bp BamHI (blunt end repaired with T4 polymerase)-*Not*I fragment was isolated from pHA.DX3 and ligated to *Sal*I (blunt end repaired with T4 polymerase) -*Not*I digested plasmid pGEX-5X-2 (GE Health Care) creating plasmid pGST.DDX3.

j) *pC.pVIIIhav.* A 693bp fragment was amplified by PCR using primers hpVIII-F and hpVIII-R (Table 5.2.1), and plasmid pH5-R (Zakhartchouk et al., 1999) DNA as a template. The PCR product was digested with *Bam*HI - *Xho*I and ligated to *Bam*HI- *Xho*I digested plasmid pcDNA.3HA creating plasmid pC.pVIIIhav.

k) *pC.pVIIIpav.* A 681bp fragment was amplified by PCR using primers PpVIII-F and PpVIII-R (Table 5.2.1), and plasmid pGADT7-pVIII (Singh et al., 2005) DNA as a template. The PCR fragment was digested with *Bam*HI- *Eco*RI and ligated to *Bam*HI-*Eco*RI digested plasmid pcDNA.3HA creating plasmid pC.pVIIIpav.

5.2.4. Yeast II hybrid assay

The Matchmaker two hybrid system3 using Saccharomyces cerevisiae (Clontech) was used to detect the interactions between pVIII and DDX3 as described (Kulshreshtha and Tikoo, 2008). The pVIII gene was cloned in-frame to GAL4 DNA binding domain (pGB.pVIII) or GAL4 activation domain (pGA.pVIII). Similarly, DDX3 gene was cloned in-frame to GAL4 DNA binding domain (pGB.DX3) or in-frame to GAL4 activation domain pGA.DX3). Yeast AH109 cells (Clontech) were co-transformed with either pGA.DX3 or pGB.pVIII or pGA.pVIII and pGB.DX3 plasmid DNAs. The growth and selection of positive clones was performed as described earlier (Kulshreshtha and Tikoo, 2008; Singh et al., 2005). DNA was isolated from positive clones and sequenced for confirmation.

5.2.5. Recombinant protein expression and protein purification

The recombinant GST-fusion proteins were expressed in *Escherichia coli* BL21 cells as described (Kulshreshtha and Tikoo, 2008). Glutathione S-transferase (GST), GST-pVIII and

Table 5.2.1 List of primers

Primer name	Primer Sequence
VIII-F	5’-CATATGAGCAAAGAAATTCCCACAC
VIII-R	5’-GAATTCCGCTATAACCGCTCACAG
DDX3-F	5’-GCGAATTCATGAGTCATGTGGCGGTGG
DDX3-R	5’-GCGGATCCACGTTACCCCAGTCAAC
3HA-F	5’-ATAAAGCTTCGCCACCATGGATTACCCATACGATGTTCCTGAC
3HA-R	5’-CTAGAATTCGCCCGGGCCAGCGTAATCTGGAACGTCATATGG
hp-VIII-F	5’-GCGGATCCATGAGCAAGGAAATTCCCACGCCCTAC
hpVIII-R	5’-ATCTCGAGTCATCAGTCGTAGCCGTCCGCCGAGTC
PpVIII-F	5’-GCGGATCCATGAGCAAACAAATCCCCAC
PpVIII-R	5’-CGAATTCTCAGTCATAGCCTGCTACAGAGTCCG
Fir-F	5’- CGTGCCAGAGTCTTTCGACA
Fir-R	5’- ACAGGCGGTGCGATGAG

GST-DDX3 fusion proteins were purified using Glutathione sepharose beads (GE Health care) as per the instructions of the manufacturers. The purified proteins were dialyzed using Slide-A-Lyzer dialysis cassette (Thermo Scientific). The concentrations of the proteins were measured by Bradford assay (Bio Rad) using Ultrospec® 3000 spectrophotometer (Pharmacia Biotech).

5.2.6. *In-vitro* transcription, translation and Co-immunoprecipitation assay

Individual plasmid DNAs (0.8µg) were used to synthesize [^{35}S] (Perkin Elmer) labeled human adenovirus (HAdV)-5 pVIII (pC.pVIIIhav) and porcine adenovirus (PAdV)-3 pVIII (pC.pVIIIpav) proteins or unlabeled DDX3 (pcHA.DX3) *in-vitro* by utilizing a TNT T7 Coupled Reticulocyte Lysate System (Promega). Equal amounts of proteins (unlabeled DDX3 and labeled HAdV-5 pVIII or unlabeled DDX3 and labeled PAdV-3 pVIII) were mixed together and incubated for 4-6hrs at 4^{o}C. The individual mixture was immunoprecipitated either with anti-DDX3 serum or rabbit pre-immune sera coupled to protein A sepharose beads. The bound proteins were separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), fixed in de-stain solution (200ml methanol, 100ml glacial acetic acid and 700ml dd$H_2$0) for 30min and dried. Subsequently, the gel was exposed to a phosphor screen (Kodak) and visualized on a Molecular Imager FX using Quantity One software (Bio-Rad).

5.2.7. *In-vitro* binding assay

pHA.DX3 (0.8µg) was used to synthesize radio-labeled DDX3 protein *in-vitro* by utilizing a TNT T7 Coupled Reticulocyte Lysate System (Promega) in the presence of 30µCi of [^{35}S]-methionine (Perkin Elmer). Prior to use, glutathione sepharose (GST) beads were washed three times with GST binding buffer (0.54M NaCl, 2.7mM KCl, 10.15mM Na2HPO4, 1.75mM

KH2PO4, 0.01M MgCl2, 1µg/ml each Aprotinin and Leupeptin, 1% Triton X-100, 1mM PMSF and DNAse I). Purified GST, GST-pVIII or GST-DDX3 fusion protein (15µg each) was incubated individually with 20µl of glutathione sepharose beads (GE Health Care) plus 10µl of *in-vitro* synthesized indicated proteins at +4^{o}C on a nutator. After overnight incubation, the beads were washed three times, 10min each with 0.1M PBS. The bound proteins were analyzed as described above.

5.2.8. Immunoprecipitation and Western blot

MDBK cells were infected with BAdV-3 at an MOI of 5. Vero cells (6 x 10^5/well) in 1 well of 6 well plate were co-transfected with 2µg of each plasmid (pHA.DX3 and pEY.pVIII or pHA.DX3 and pEYFPN1) DNA. At 36hrs post infection or 48hrs post transfection, the cells were lysed, immunoprecipitated with indicated protein specific antibodies, separated by 10% SDS-PAGE, transferred to PVDF membrane and probed by Western blot using indicated protein specific antibodies as described (Poulin et al., 1998).

5.2.9. Confocal microscopy

Monolayers (60-70% confluent) of Vero cells (5x10^4/well) in two well chamber slides were incubated with Opti-MEM I reduced serum media (Gibco). After 2hrs of incubation, the cells were transfected with indicated plasmid DNA(s) (1-2µg/plasmid DNA/well) using Lipofectamine 2000 (Invitrogen). After 36hrs post transfection, the cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.05% Triton X-100 and blocked with 2% goat serum. Subsequently, the cells were incubated with protein specific antibody in 0.1M PBS for 1hr at room temperature**,** followed by specific secondary antibody for 1hr. Finally, the slides were

mounted with mounting medium containing DAPI (Vectashield) and visualized under Zeiss LSM 5 laser scanning confocal microscope.

Monolayers (90% confluent) of MDBK cells ($1x10^5$cells/well) grown on two well chamber slides were mock infected or infected with wild type BAdV-3 at an MOI of 5. At 24hrs post infection, the cells were fixed with 3.7% paraformaldehyde, immunostained and visualized as described above.

5.2.10. *In-vitro* synthesis and translation of capped and uncapped mRNA

Firefly luciferase mRNAs were synthesized *in-vitro* in the absence (IRES) or presence (capped) of 40mM Ribo m7GpppG cap analogue (Promega) using T7-RiboMax RNA production system. 10µl of prewashed GST-beads were loaded with different concentrations of (50, 100, 250, 350, 550, 750ngs) of either GST-pVIII or GST proteins and incubated for 2hrs at $+4^oc$ with 15µl of rabbit reticulolcyte lysate. Subsequently, the mixtures were centrifuged and the supernatants were used for the translation of the synthesized capped and uncapped mRNAs. Finally, luciferase assay (Promega) was performed in a luminometer as per the company's procedure.

5.2.11. Dual luciferase assay

To examine the effect of pVIII on the translation of IRES (Polio virus IRES) or Cap dependent translation in 293T cells, a bicistronic luciferase assay was performed using a plasmid DNA expressing a single transcriptional unit in which translation of renilla luciferase is cap-dependent while translation of firefly luciferase is cap-independent (IRES dependent)**.** The cells grown in six well plates were co transfected with 2µg/well of a bicistronic reporter plasmid

pcDNA3-RLuc-POLIRES-FLuc and 4µg/well of either plasmid pEY.pVIII DNA or plasmid pEYFP N1 DNA. At 48hrs post transfection, Firefly luciferase (FLuc) and Renilla reniformis luciferase (RLuc) activities were measured in a luminometer by using a dual luciferase assay kit (Promega) as per the company's procedure. Expression of EYFP was used to normalize the transfection efficiency.

5.2.12. Cellular protein synthesis assay

The monolayers of MDBK cells in 6 well plates were mock infected or infected with BAdV-3 at a MOI of 5 or monolayers of Vero cells grown in 6 well plates were transfected with indicated concentrations of plasmid pEY.pVIII or pEYFPN1. At different times post transfection, the cells were starved in media without methionine for 2hrs before labeling with 100 µCi of [^{35}S] methionine for 10min. The radiolabelled cells were collected and lysed with RIPA buffer containing protease inhibitor cocktail. Proteins from radiolabelled cell lysates were separated by 10% SDS-PAGE. The gel was fixed, exposed to a phosphor screen (Kodak) overnight and visualized on a Molecular Imager FX using Quantity One software (Bio-Rad).

5.2.13. Analysis of DDX3 and eIFs *in-vitro*

To determine whether the reduction in the level of the translation of capped luciferase mRNA in the presence of pVIII is due to the reduction in the level of eIFs, initially 10µl of Flexi rabbit reticulo Lysate was incubated with 10µl of GST bead preloaded with 750ng of either GST or GST-pVIII fusion protein at +4^{o}C and centrifuged for 10min. Finally, the supernatant and pellet were collected and analyzed by Western blot using anti-DDX3 or anti -eIF specific antibodies, followed by Alexa Flour 680 goat anti rabbit IgG (Molecular Probes) or IRDye 800

conjugated goat-anti-mouse IgG (Li-COR biosciences) as secondary antibody. The membranes were scanned using Odyssey LiCOR infrared scanning system and the intensity of the bands measured by Odyssey Software v2.1. Similarly, 50µl of the cytoplasmic fraction of cells were incubated with 10µl of GST beads (loaded with 750ng of either GST or GST-pVIII fusion protein) at +4^{o}C and centrifuged for 10min. Finally, the samples were analyzed by Western blot as described above.

5.2.14. 7 -Methyl Guanosine Cap binding assay

MDBK, VIDO R2 or DDX3 negative (DDXshRNA knockdown) VIDO R2 cells grown on 6 well plates were infected with 5 MOI of BAdV-3. 293T cells grown on 6 well plates were transfected either with 2µg/well of plasmid pEYFPN1 or pEY.pVIII DNAs. At 36hrs post infection or transfection, the cells were lysed with RIPA buffer containing protease inhibitor cocktail and centrifuged at 10,000rpm. The supernatants were incubated with 50µl of 7-methyl GTP sepharose beads (GE Healthcare) on a nutator overnight at +4^{o}c. The beads were then washed three times five minutes each and boiled with protein loading dye with 10% beta mercaptoethanol. Proteins were separated by 10%SDS-PAGE gel, transferred to nitrocellulose and probed in Western blot using anti- DDX3 or anti-eIF specific antibody followed by Alexa Flour 680 goat anti rabbit IgG (Molecular Probes) or IRDye 800 conjugated goat anti-mouse IgG (Li-COR biosciences) as secondary antibody. The expression of pVIII in BAdV-3 infected cells or EY.pVIII in transfected cells was checked by Western blot using anti-pVIII serum.

5.2.15. Electrophoretic mobility shift assay (EMSA)

Confluent MDBK cells grown in 6 well plates were starved with media without phosphate for 1hr before radiolabelling with 30µciof [^{32}P] UTP (Perkin Elmer, Cat # BLU007H250UC, 3000Ci/mml). After labeling for 1 hr, the cytoplasmic RNA was extracted using SurePrep^{TM} nuclear or cytoplasmic RNA purification kit (Fisher Bioreagents). Finally, poly A^{+} RNA was purified using Oligotex® mRNA mini kit (Qiagen). The purity of the RNA was further checked by digestion with either RNase or DNase enzymes followed by gel electrophoresis. Subsequently, RNA EMSA was carried out using a modified protocol by Yakhnin et al, 2012. Radiolabeled (10,000 cpm) RNA probe of oligo dt purified polyA RNA was incubated with 500ng of GST alone, or GST fusion proteins GST.pVIII or GST.100K (BAdV-3 100K) plus 10µg yeast tRNA (Ambion) at room temperature. After incubation for 30 mins, the reaction mixture was separated by 4% acrylamide native gel for 4-6 hr at 150 V before the gel was dried, exposed onto a phosphor screen (Kodak) and scanned on a Molecular Imager FX using Quantity One software (Bio-Rad).

5.2.16. Real time PCR

MDBK cells were mock infected or infected with wild type BAdV-3 at an MOI of 5. The cells were collected at different times post infection and cytoplasmic RNA was extracted using SurePrep^{TM} nuclear or cytoplasmic RNA purification kit. cDNA was synthesized using Superscript II (Invitrogen) followed by real time PCR using SYBR green and primers targeting different species of bovine housekeeping genes (Lisowski et al., 2008). pcDNA3-RLuc-POLIRES-FLuc and pEY.pVIII or EYFP NI plasmid transfected 293T cells were collected at 36hrs post transfection and RNA was extracted using SurePrep^{TM} nuclear or cytoplasmic RNA

purification kit. cDNA was synthesized using Superscript II (Invitrogen) followed by real time PCR using SYBR green and primers targeting RLuc-POLIRES-FLuc mRNA using primers Fir-F and Fir-R (Table 5.2.1)

5.3. RESULTS

5.3.1. Interaction of BAdV-3 pVIII with cellular proteins

Initially, proteomic analysis of the nucleoli of BAdV-3 infected and uninfected MDBK cells identified a number of nucleolar proteins, which were hypothesized to be involved in BAdV-3 infection (Patel and Tikoo, unpublished data). Since one of these proteins was DDX3 we hypothesized it to be important for BAdV-3 replication. We initially determined if DDX3 interacted with any BAdV-3 protein using matchmaker GAL4 Yeast two hybrid assays. Our results indicated that BAdV-3 pVIII interacted with DDX3 (Figure 5.3.1). The interactions were confirmed by GST-pull down assays (Figure 5.3.2A) and co immunoprecipitation assays using transfected cells (Figure 5.3.2B, C). Moreover, confocal microscopy also suggested that pVIII co-localized with DDX3 around the perinuclear region and in the cytoplasm of transfected cells (Figure 5.3.3 panel h1-h4) or BAdV-3 infected cells (Figure 5.3.3 panel g1-g4). However, DDX3 localized diffusely in the cytoplasm in uninfected MDBK or untransfected Vero cells (Figure 5.3.3 panels a & b). Plasmid DNA expressed DDX3 also localized diffusely in the cytoplasm (Figure 5.3.3 Panel c). Additionally, pVIII is localized both in the cytoplasm and in the nucleus in BAdV-3 infected (Figure 5.3.3 panel e) or pEY-VIII transfected (Figure 5.3.3 panel f) cells. No pVIII was detected in uninfected MDBK cells (Figure 5.3.3 panel d).

Finally, using co-immunoprecipitation coupled with Western blot analysis, we determined the interaction of DDX3 with pVIII in BAdV-3 infected MDBK cells. As seen in Figure 5.3.2, DDX3 specific protein could be detected in the lysates of BAdV-3 infected MDBK

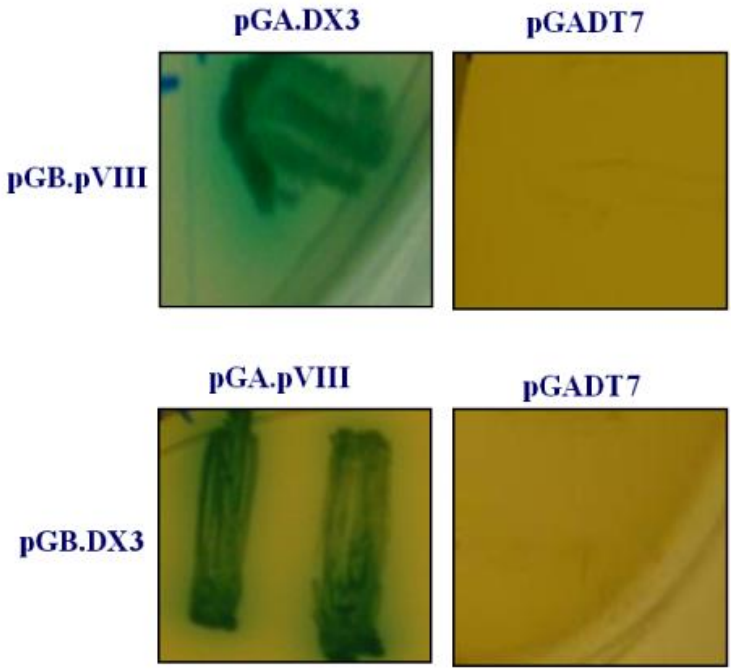


Figure 5.3.1. Yeast two hybrid analysis. The AH109 yeast cells were co-transfected with plasmid (pGB.pVIII and pGA.DX3; pGB.pVIII and pGADT7; pGB.pDX3 and pGA.pVIII or pGB.DX3 and pGADT7) DNAs and streaked on a selective medium containing X-α-gal but lacking Leu, Trp, His and Ade.

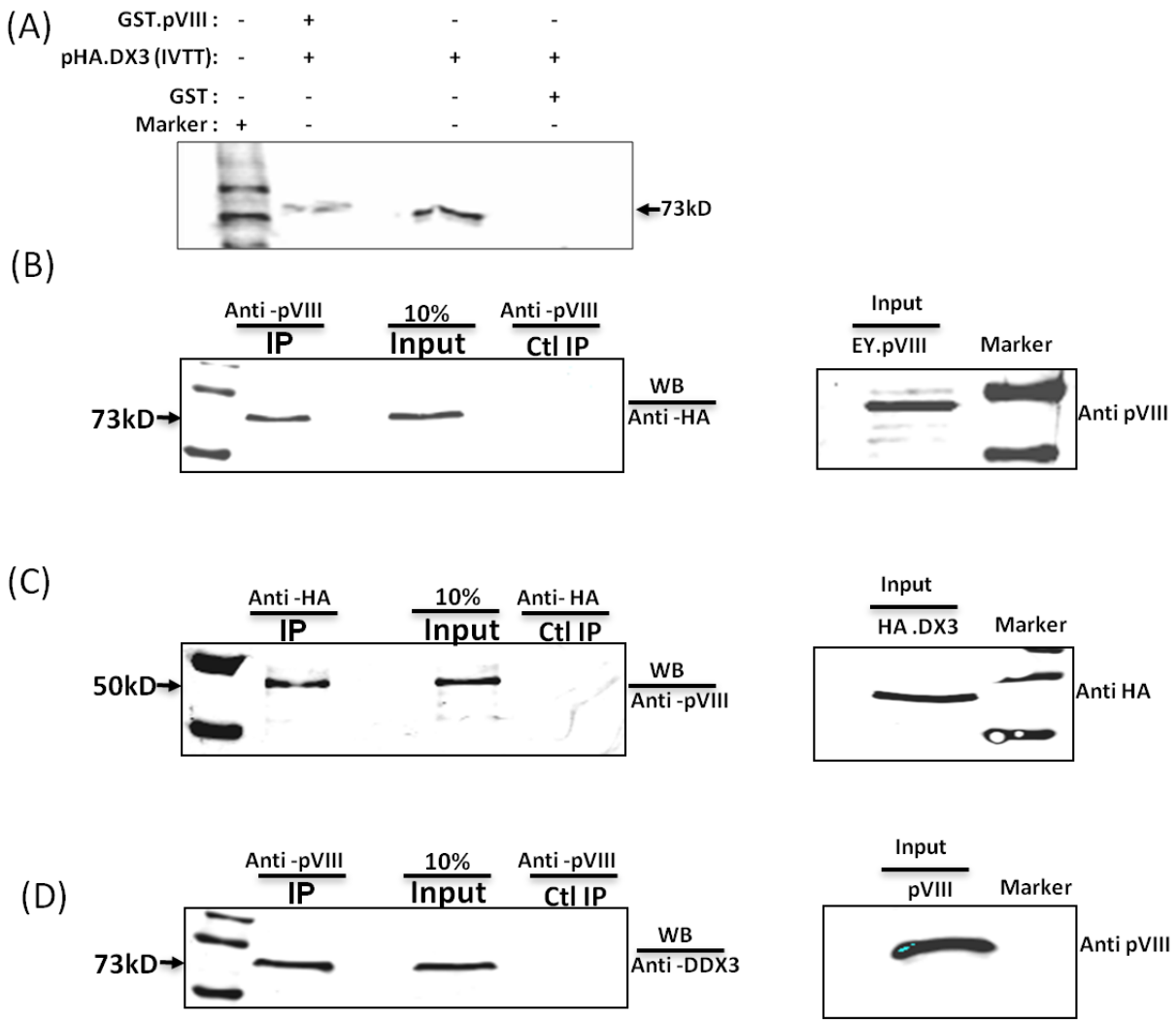

(A)
GST.pVIII : - + - -
pHA.DX3 (IVTT): - + + +
GST : - - - +
Marker : + - - -
73kD
(B)
Anti -pVIII
IP
10%
Input
Anti -pVIII
Ctl IP
73kD
WB
Anti -HA
Input
EY.pVIII
Marker
Anti pVIII
(C)
Anti -HA
IP
10%
Input
Anti- HA
Ctl IP
50kD
WB
Anti -pVIII
Input
HA .DX3
Marker
Anti HA
(D)
Anti -pVIII
IP
10%
Input
Anti -pVIII
Ctl IP
73kD
WB
Anti -DDX3
Input
pVIII
Marker
Anti pVIII

Figure 5.3.2 Interaction of DDX3 with BAdV-3 pVIII. (A) GST pull down assay. Purified GST or GST-pVIII fusion protein immobilized on Glutathione-Sepharose 4B beads, incubated with in-vitro translated [^{35}S] methionine labeled HA tagged DDX3 were separated by 10% SDS-PAGE and detected by autoradiography. **(B &C)** Co-immunoprecipitation in transfected cells. Proteins from the lysates of cells co-transfected with either pHA.DX3 and pEY.pVIII or pHA.DX3 and pEYFPN1 were immunoprecipitated with anti-pVIII serum **(B)** or anti-HA **(C),** separated by 10% SDS-PAGE and transferred to nitrocellulose membrane. The separated proteins were probed in Western blot using anti-HA MAb **(B)** or anti-pVIII serum **(C)**. **(D)** Co-immunoprecipitation in BAdV-3 infected cells. Proteins from the lysates of mock or BAdV-3 infected MDBK cells were immunoprecipitated with anti-pVIII serum, separated by 10% SDS-PAGE, transferred to nitrocellulose membrane and probed in Western blot using anti-DDX3 MAb. Immunoprecipitation (IP). Western blot (WB).

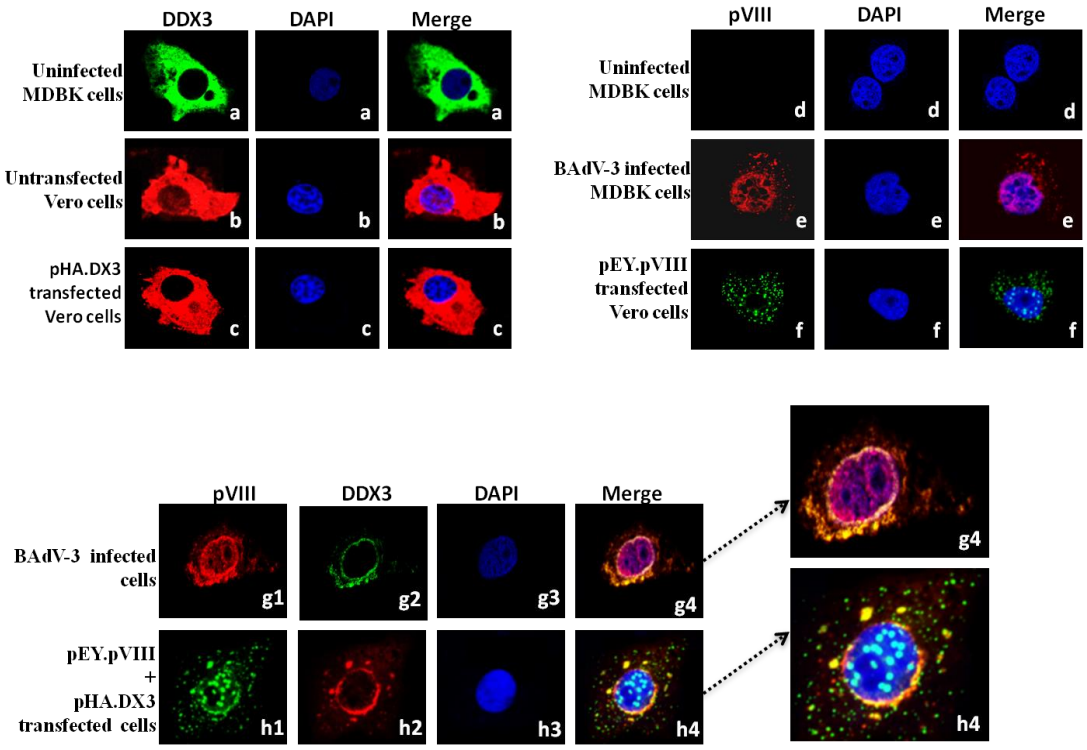


Figure 5.3.3 Confocal microscopy. MDBK cells mock infected (panel a, d) or infected with BAdV-3 (panels e, g) VERO cells untransfected (panel b) or transfected with indicated plasmid (panels c, f, h) DNA, were fixed 36hrs post-infection/transfection. The subcellular localization of DDX3 (panels a,b,c,g2,h2) protein was visualized by indirect immunofluorescence (panels a,b,c,g2,h2) using anti-DDX3 MAb and fluorescein conjugated goat anti mouse IgG-FITC (panels a, g2), anti-DDX3 MAb and Cy3 conjugated goat anti -mouse (pane b) secondary antibody, anti-HA MAb and Cy3 conjugated goat anti -mouse (pane b) secondary antibody (panel c,h2). The subcellular localization of pVIII (panels d,e,f,g1,h1) was visualized by direct fluorescence (panels f,h1) or indirect immunfluorescene using anti-pVIII serum and Cy3

conjugated goat anti -rabbit secondary antibody (panels d,e,g1). Nuclei were stained with DAPI in each panel. A merge of the images is shown. Enlarged panel g4 and h4 is shown with arrows. cells immunoprecipitated with anti- pVIII serum and probed in Western blot with anti-DDX3 MAb (panel D).

5.3.2. Interaction of DDX3 with pVIII of HAdV-5 and PAdV-3

To verify if interaction of DDX3 with pVIII is conserved among members of *Mastadenovirus* genus, purified GST or GST-DDX3 (Figure 5.3.4A) fusion protein bound to Sepharose beads was individually incubated with *in-vitro* transcribed and translated radio-labeled [^{35}S] HAdV-5 pVIII or PAdV-3 pVIII. After washing, the bound proteins were separated on 10% SDS-PAGE and visualized by autoradiography. As seen in Figure 5.3.4B, radiolabelled HAdV-5 pVIII or PAdV-3 pVIII protein interacted with GST-DDX3 fusion protein but not with GST protein alone.

Next, we performed co-immunoprecipitation assay to corroborate the results of GST pull down assay. The *in-vitro* translated unlabeled DDX3 was incubated with *in-vitro* translated radiolabeled HAdV-5 pVIII or PAdV-3 pVIII for 4-6 hr at 4^{o}C before immunoprecipitating with antibodies against DDX3. The co-immunoprecipitated radiolabeled proteins were separated on 10% SDS-PAGE and visualized by autoradiography. As seen in Figure 5.3.4C, radiolabeled HAdV-5 pVIII or PAdV-3 pVIII co-immunoprecipitated with unlabeled DDX3 using anti-DDX3 serum.

5.3.3. BAdV-3 pVIII affects cellular protein synthesis

To determine if BAdV-3 infection inhibits the synthesis of cellular proteins at late times post infection, mock or BAdV-3 infected MDBK cells were pulsed for short duration with [^{35}S] methionine at different times post infection. Proteins from the lysates of the labeled cells were

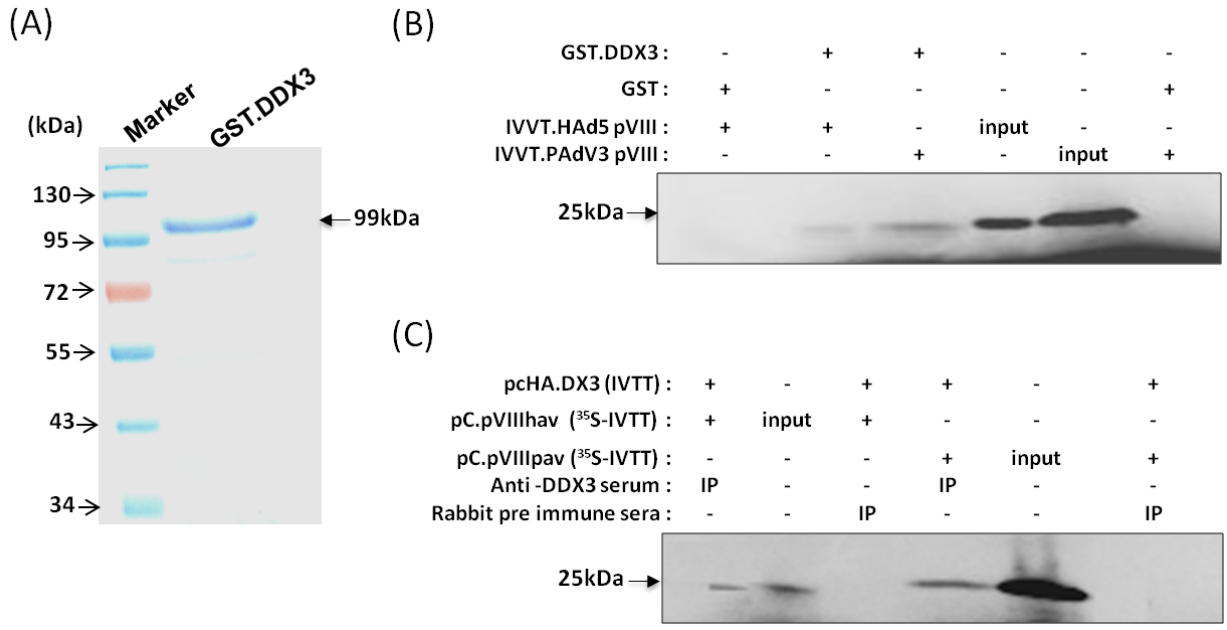


Fig. 5.3.4 Interaction of DDX3 with PAdV-3 & HAdV-5 pVIII. (A) Coomassie blue staining of GST.DDX3 protein. **(B)** GST-pull down assay. Purified GST or GST-DDX3 fusion protein immobilized on Glutathione-Sepharose 4B beads, incubated individually with *in-vitro* translated [^{35}S] methionine labeled PAdV-3 pVIII or HAdV-5 pVIII, separated by 10% SDS-PAGE and detected by autoradiography. **(C)** Co-immunoprecipitation. Radio labeled in-vitro transcribed and translated HAdV5 pVIII or PAdV-3 pVIII was incubated with in-vitro transcribed and translated unlabelled DDX3 protein. Proteins were immunoprecipitated with either anti-DDX3 serum or rabbit pre immune sera, separated by 10% SDS-PAGE and autoradiographed. Immunoprecipiation (IP).

separated by 10% SDS-PAGE and analyzed by autoradiography. As seen in Figure 5.3.5A, compared to mock infected MDBK cells, reduction in cellular protein synthesis is apparent in BAdV-3 infected cells at 18hrs post infection, which coincides with the detection of expression of pVIII (a late protein) in BAdV-3 infected cells. By 36hrs to 48hrs post infection, there is significant decrease in cellular protein synthesis, which coincides with steady expression of pVIII protein (Figure 5.3.5A).

To determine if expression of pVIII alters the cellular protein synthesis, Vero cells were transfected with indicated plasmid DNA, labeled with [^{35}S] methionine, and analyzed by SDS-PAGE. As seen in Figure 5.3.5B, there was noticeable reduction in the synthesis of cellular protein in cells transfected with plasmid pEY.pVIII DNA than in cells transfected with plasmid pEYFPN1 DNA at 24hrs to 48hrs post transfection. Moreover, the reduction in cellular protein synthesis appeared to depend on the amount of plasmid DNA used for transfection (Figure 5.3.5C).

5.3.4. BAdV-3 pVIII affects translation of capped mRNAs

To determine the effect of pVIII on mRNA translation, mRNAs were synthesized *in-vitro* in the absence (IRES) or presence (capped) of 40mM Ribo m7GpppG cap analogue (Promega) using T7-RiboMAX RNA production system. The synthesized mRNAs (Figure 5.3.6A) were translated in the presence of purified GST or fusion protein GST.pVIII. As seen in Figure 5.3.6A, GST-pVIII protein significantly inhibited the *in-vitro* translation of capped but not of

uncapped luciferase mRNA. No such effect could be observed in the presence of GST on the translation of capped or uncapped luciferase mRNAs.

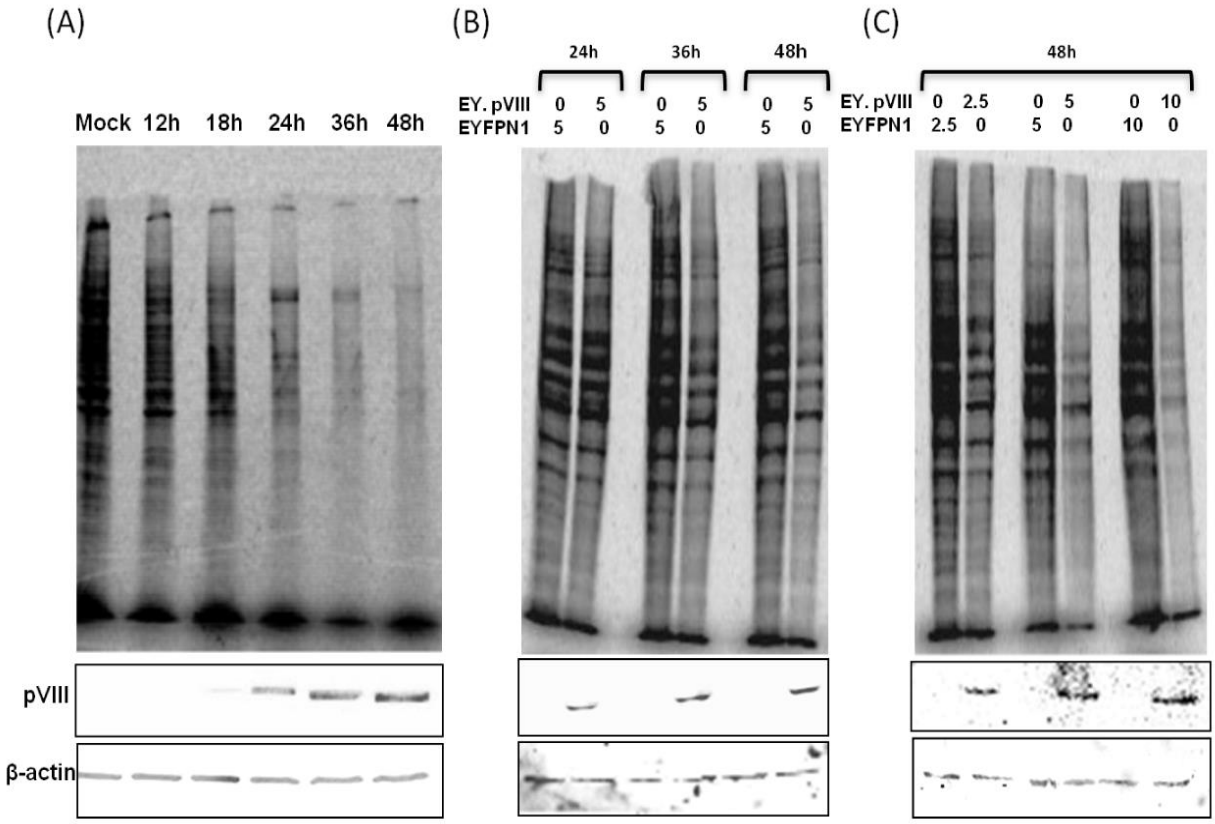


Figure 5.3.5 Protein synthesis in BAdV-3 infected cells. (A) Monolayers of MDBK cells were mock infected or infected with BAdV-3 at a MOI of 5. **(B &C)** Monolayers of VERO cells were transfected with indicated amounts of plasmid DNAs. At indicated times post infection **(A)** or transfection **(B, C),** the cells were pulse labeled with [^{35}S] methionine for 10mins. The radiolabelled proteins were separated by 10% SDS-PAGE and analysed by autoradiography. Proteins from the lysates of radiolabeled cells **(A, B or C)** were subjected to SDS-PAGE and Western blot using anti-pVIII serum, or anti β-actin MAb.

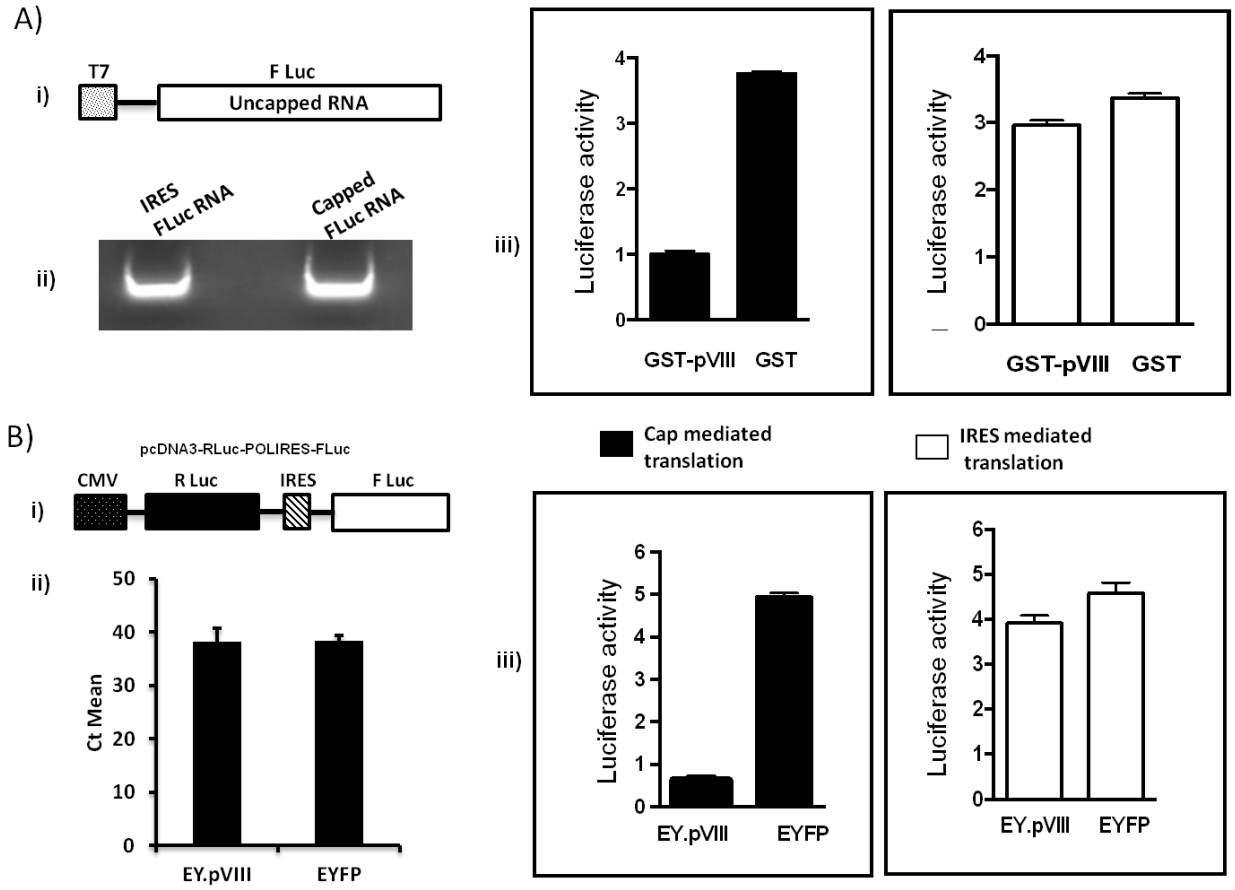

A)
i)
T7
F Luc
Uncapped RNA
IRES FLuc RNA
Capped FLuc RNA
ii)
iii)
Luciferase activity
GST-pVIII
GST
B)
pcDNA3-RLuc-POLIRES-FLuc
CMV
R Luc
IRES
F Luc
i)
ii)
Ct Mean
EY.pVIII
EYFP
Cap mediated translation
IRES mediated translation
iii)
Luciferase activity
EY.pVIII
EYFP

Figure 5.3.6 Effect of pVIII on capped mRNA translation. (A). *In-vitro*. The TNT® T7 luciferase DNA (Promega) (i) was transcribed *in-vitro* in the absence (IRES) or presence (capped) of 40mM Ribo m7GpppG cap analogue (Promega) using RiboMAX RNA production system-T7 (Promega). The *in-vitro* synthesized capped and uncapped luciferase mRNAs (ii) were translated in the supernatant collected after centrifugation of mixture of Flexi Rabbit Reticulo Lysate (Promega) incubated with Glutathione sepharose beads preloaded with GST-VIII or GST protein alone. The level of luciferase activity was measured using a luciferase kit (Promega) on a Luminometer (Turner Designs Inc). The results are shown as relative luciferase activity (iii) **(B)** *In-vivo*. 293T cells were transfected with plasmid DNAs (2µg of pcDNA3-RLuc-POLIRES-FLuc (i) and either 4µg of pEY.pVIII or 4µg of pEYFPN1). At 36hrs post transfection, Firefly luciferase (FLuc) and Renilla reniformis luciferase (RLuc) activities were measured in a luminometer by using a dual luciferase assay kit (Promega) as per the company's procedure. Expression of EYFP was used to normalize the transfection efficiency. The results are shown as luciferase activity (iii). The level of cytoplasmic RLuc-POLIRES-FLuc mRNA both in EY.pVIII and EYFP expressing plasmid transfected cells was quantified by RT-PCR (ii).

To examine the effect of BAdV-3 pVIII on the translation of capped and uncapped mRNA translation *in-vivo*, a bicistronic luciferase assay was performed. The 293T cells were co-transfected with plasmid pcDNA3-RLuc-POLIRES-FLuc (producing a single transcriptional unit in which the translation of renilla luciferase is cap-dependent while translation of firefly is cap independent [IRES dependent]) DNA and either plasmid pEY-pVIII or plasmid pEYFPN1 DNA using a dual luciferase assay kit (Promega) as per the company's procedure. As shown in Figigure 5.3.6B, in the presence of EY-pVIII, the cap dependent translation of renilla luciferase mRNA (capped) was significantly reduced compared to IRES dependent translation of firefly luciferase mRNA (capped) in 293T cells. In contrast, there was no significant difference in the translation of renilla luciferase (cap-dependent) mRNA or firefly luciferase (IRES dependent) in 293T cells in the presence of EYFP protein (Figure 5.3.6B). Moreover, cytoplasmic RLuc-POLIRES-FLuc mRNA levels appeared similar in EY.pVIII or EYFP expressing plasmid transfected 293T cells (Figure 5.3.6B).

5.3.5. pVIII reduces the level of DDX3 and translation initiation factors (eIFs) from cap binding complex

To determine if the interaction of pVIII and DDX3 also affects the level of eIFs, the pellet and supernatant fractions of the rabbit reticulocyte lysate incubated with glutathione sepharose beads preloaded with purified GST-pVIII or GST were analyzed by Western blot using protein specific antibodies. As seen in Figure 5.3.7A, addition of GST-pVIII in reticulo lysate reduced the amount of DDX3, eIF3β, eIF4G and PABP in supernatant fraction relative to

the amount of DDX3, eIF3, eIF4G or PABP in supernatant fraction when purified GST alone was added to reticulo Lysate (Figure 5.3.7A). Similarly, addition of GST-pVIII in cytoplasmic

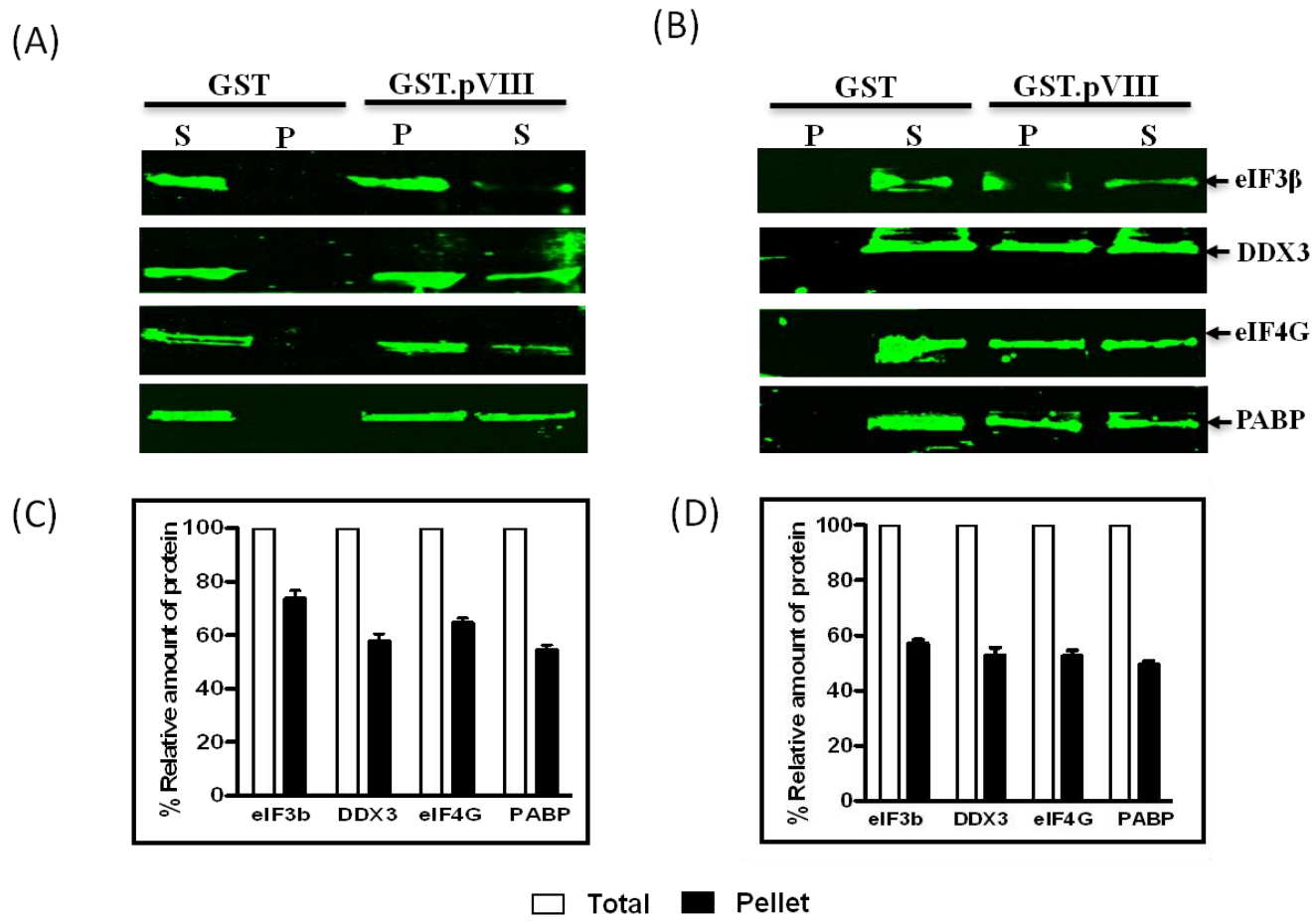


Figure 5.3.7. The effect of pVIII on levels of eIFs. The cytoplasmic fraction of MDBK cells (50µl) or Flexi Rabbit Reticulo -Lysate (10µl) were incubated with either 10µl of GST beads loaded with 750ng of purified GST-VIII protein or 10µl of GST beads loaded with 750ng of purified GST protein for 2hrs at +4^{o}C and centrifuged for 10min. The supernatant and the pellet from both reticulo lysate **(A,C)** or cytoplasmic fraction **(B,D)** were separated by 10% SDS-PAGE gel and analyzed by Western blot using indicated protein specific antibodies and Alexa Flour 680 goat anti-rabbit IgG antibody or IRDye 800 conjugated goat anti-mouse IgG as secondary antibody. The intensity of the bands was analyzed by Odyssey Software v2.1. Error bars **(C, D)** indicate S.E of means for three separate experiments. Total amount of indicated protein in cytoplasmic fraction of MDBK cells (panel D) or reticulo-lysate (panel C) was estimated by Western blot analysis of parallel sample before adding GST or GST.pVIII fusion protein. The amount of each protein retained in the beads (pellet) relative to the total amount is plotted. Supernatant (S); Pellet (P).

fraction of MDBK cells reduced the amount of DDX3, eIF3β, eIF4G and PABP in supernatant fraction (Figure 5.3.7B) compared to the amount of DDX3, eIF3β, eIF4G and PABP in supernatant fraction when purified GST alone was added to cytoplasmic fraction of MDBK cells (Figure 5.3.7B). The amount of each protein retained in the beads (pellet) relative to the total amount is plotted (Figure 5.3.7C & D).

5.3.6. BAdV-3 infection or pVIII expression reduces the level of DDX3 and translation eIFs *in-vivo*

To determine the effect of BAdV-3 on the constituents of cap binding complex, the cleared cell lysates from mock infected or BAdV-3 infected MDBK cells collected at 36hrs post infection were incubated individually with 7-methyl GTP-Sepharose beads (GE Healthcare). The captured components of cap-binding complex were identified by Western blot using protein specific antibodies. As seen in Figure 5.3.8, no significant difference could be observed in the level of eIF4G in mock or BAdV-3 infected cells. However, significant decrease in the levels of DDX3, eIF3, eIF4E and PABP proteins was observed in BAdV-3 infected cells compared to mock infected cells. However, pVIII was not retained in the 7-methyl GTP resins. Moreover, anti-pVIII serum detected the expression of pVIII in BAdV-3 infected cells.

On the other hand, to determine if interaction of BAdV-3 pVIII with DDX3 affects the constituents of cap binding complex, the cleared lysates from pVIII expressing plasmid transfected cells collected at 36hrs post transfection were incubated separately with 7-methyl GTP-Sepharose beads. The captured components of cap-binding complex were identified by

Western blot using protein specific antibodies. As seen in Figure 5.3.8, no significant difference could be observed in the level of eIF4G in cells transfected with plasmid pEY.pVIII or plasmid

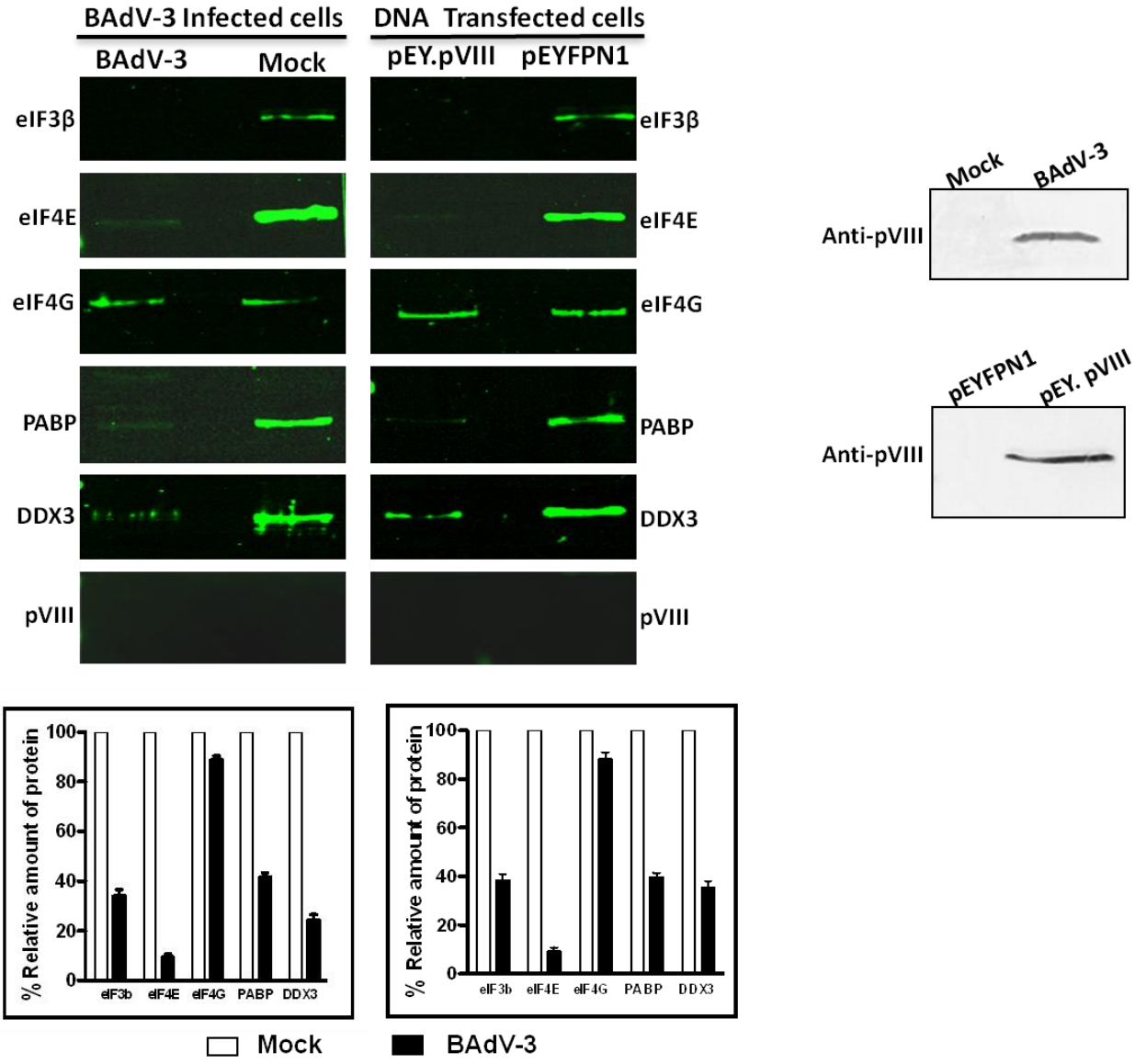


Figure 5.3.8 m7GTP-sepharose binding assay. The supernatant of the lysates of the cells collected at 36 hrs post BAdV-3 infection of MDBK cells (mock or BAdV-3) or transfection of 293T cells with plasmid DNAs (pEY.pVIII or pEYFPN1 DNA) were incubated with m7GTP sepharose cap affinity beads. After washing, the bound proteins were analyzed by Western blot using indicated protein specific antibodies and IRDye 800 conjugated goat anti-mouse IgG or Alexa Flour 680 goat anti-rabbit IgG as secondary antibody. The intensity of the bands of the Western blot in all cases was analyzed by Odyssey Software v2.1. The relative amount of proteins in BAdV-3 infected or pEY.VIII transfected cell lysates that are retained in the 7-methyl GTP resins as compared to mock infected or pEYFPN1 transfected cells, respectively (i.e. considering the amount of protein in mock infected or pEYFPN1 transfected cell lysates that are retained in the 7-methyl GTP resins as 100%) is plotted. Error bars indicate S.E of means for three separate experiments. Proteins from the lysates of BAdV-3 infected or transfected cells were separated by 10% SDS-PAGE and probed in Western blot using anti-pVIII serum.

pEYFP.NI DNA. However, there was significant decrease in the levels of DDX3, eIF3, eIF4E and PABP proteins in cells transfected with plasmid pEY.pVIII DNA than the cells transfected with plasmid pEYFP.N1 DNA. Interestingly, pVIII was not retained in the 7-methyl GTP resins. The expression of EY.pVIII fusion protein in transfected cells or pVIII in infected cells was detected using anti pVIII serum.

5.3.7. pVIII does not interact directly with eIFs

To determine if pVIII interacts directly with eIFs, the cytoplasmic fractions of VIDO R2 cells (Reddy et al., 1999) and DDX3 knocked down VIDO R2 cells (Section 7) were collected, incubated with purified GST or GST-VIII, centrifuged to collect supernatant and pellet fractions and analyzed by Western blot. As seen in Figure 5.3.9A, addition of GST-pVIII reduced the amount of DDX3, eIF3β, eIF4G and PABP in supernatant of DDX3 positive VIDO R2 cells. No such decrease was observed in the amount of DDX3, eIF3β, eIF4G and PABP in supernatant fraction when purified GST-VIII was added to cytoplasmic fraction of DDX3 knocked down VIDO R2 cells.

To determine if BAdV-3 infection degrades eIFs, VIDO R2 cells were mock infected or infected with BAdV-3. At 36hrs post infection, the proteins from cell lysates were analysed by 10% SDS-PAGE using protein specific antibodies. β-actin was used as a loading control. As seen in Figure 5.3.9B, significant level of PABP, eIF3β, eIF4G, DDX3 and eIF4E could be detected in BAdV-3 infected cells.

5.3.8. Interaction of pVIII with PolyA containing mRNA

To assess the possibility of the interaction of pVIII with RNA, ^{32}P (UTP) labeled Poly A

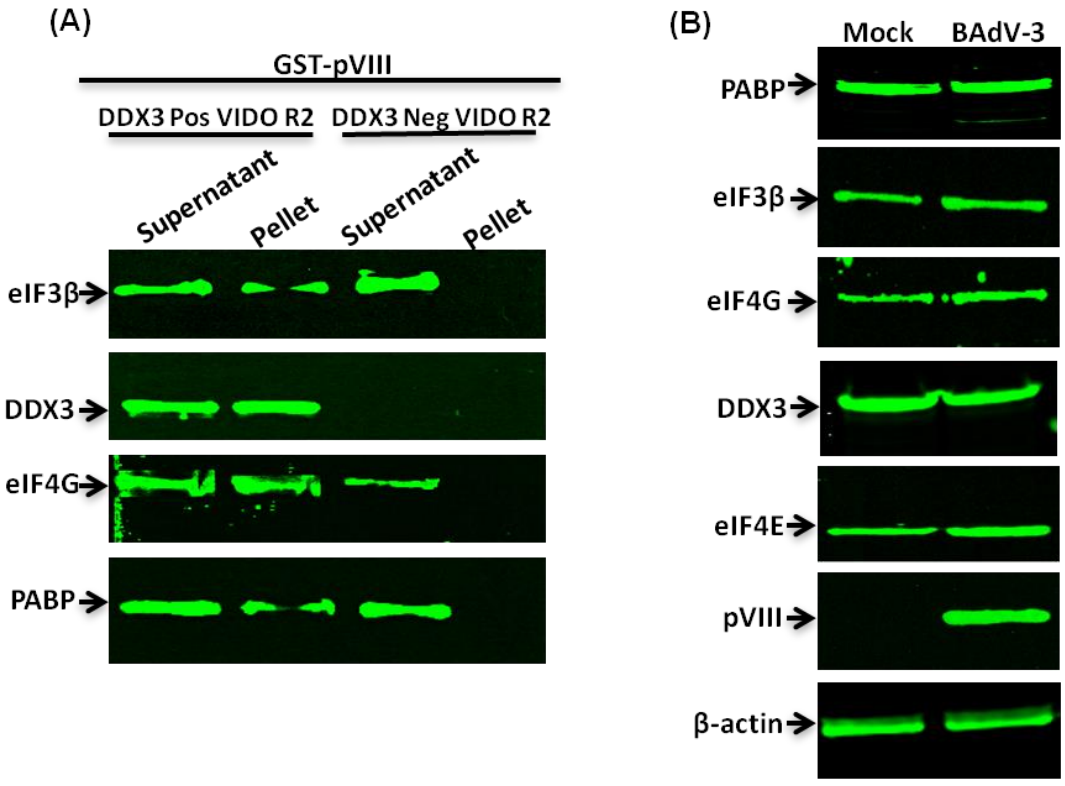

(A)
GST-pVIII
DDX3 Pos VIDO R2
DDX3 Neg VIDO R2
Supernatant
Pellet
Supernatant
Pellet
eIF3β
DDX3
eIF4G
PABP
(B)
Mock
BAdV-3
PABP
eIF3β
eIF4G
DDX3
eIF4E
pVIII
β-actin

Figure 5.3.9 Interaction of pVIII with eIFs. (A) The cytoplasmic fraction (50µl) of DDX3 positive or negative VIDO R2 cells were incubated with 10µl of beads loaded with 750ng of purified GST-VIII protein for 2hrs at 4^{o}C and centrifuged for 10min. The supernatant and the pellet from both cytoplasmic fraction were separated by 10% SDS-PAGE gel and analyzed by Western blot using indicated protein specific antibodies and IRDye 800 conjugated goat anti-mouse IgG or Alexa Flour 680 goat anti rabbit IgG as secondary antibody. Total amount of indicated protein in cytoplasmic fraction of indicated cells was estimated by Western blot analysis before adding GST.pVIII fusion protein. **(B).** Proteins from the lysates of mock infected or BAdV-3 infected MDBK cells collected at 36hrs post infection were separated by 10% SDS-PAGE and analysed by Western blot using protein specific antibody and anti-rabbit IRDye 800 conjugated goat anti-mouse IgG (Li-COR biosciences) or Alexa Flour 680 goat anti-rabbit IgG as secondary antibody. β-actin was used as a loading control.

containing mRNA was purified from the cytoplasmic fraction of MDBK cells and RNA electrophoretic mobility shift assay was performed using purified GST alone or fusion proteins GST.pVIII, GST.100K. As seen in Figure 5.3.10, GST.pVIII did not interact with the cellular poly A RNA. As expected (Cuesta and Schneider, 2004) GST.100K interacts with cellular polyA mRNA. Similarly, no interaction was observed between the cellular polyA RNA and GST.

5.3.9. Cytoplasmic mRNA levels of selected bovine genes in BAdV-3 infected cells

To examine whether cytoplasmic mRNA levels are affected or their stability compromised, cDNAs were synthesized from cytoplasmic mRNAs purified from mock infected cells or BAdV-3 infected cells collected at different time points post infection. Real time PCR was performed using primers targeting different species of bovine housekeeping genes (Lisowski et al., 2008). The results indicate that (Figure 5.3.11) as compared to mock infected cells, the level of cytoplasmic mRNAs is not altered in BAdV-3 infected cells even at 48hrs post infection Moreover, the integrity of cellular mRNAs appears intact during BAdV-3 virus infection. The detection of expression of DNA binding protein in BAdV-3 infected cell lysates by Western blot using anti-DBP serum (Zhou et al., 2001) to confirm virus infection.

5.4. DISCUSSION

DDX3 is a member of the ATPase super family II of RNA helicases (Silverma et al., 2003) and is involved in RNA metabolism (Schroder, 2011). Targeting of DDX3 by a viral protein has also established its role in TBK1/IKKε mediated IRF activation and INFβ promoter induction (Schroder et al., 2008). So far, proteins encoded by few viruses have been shown to maneuver

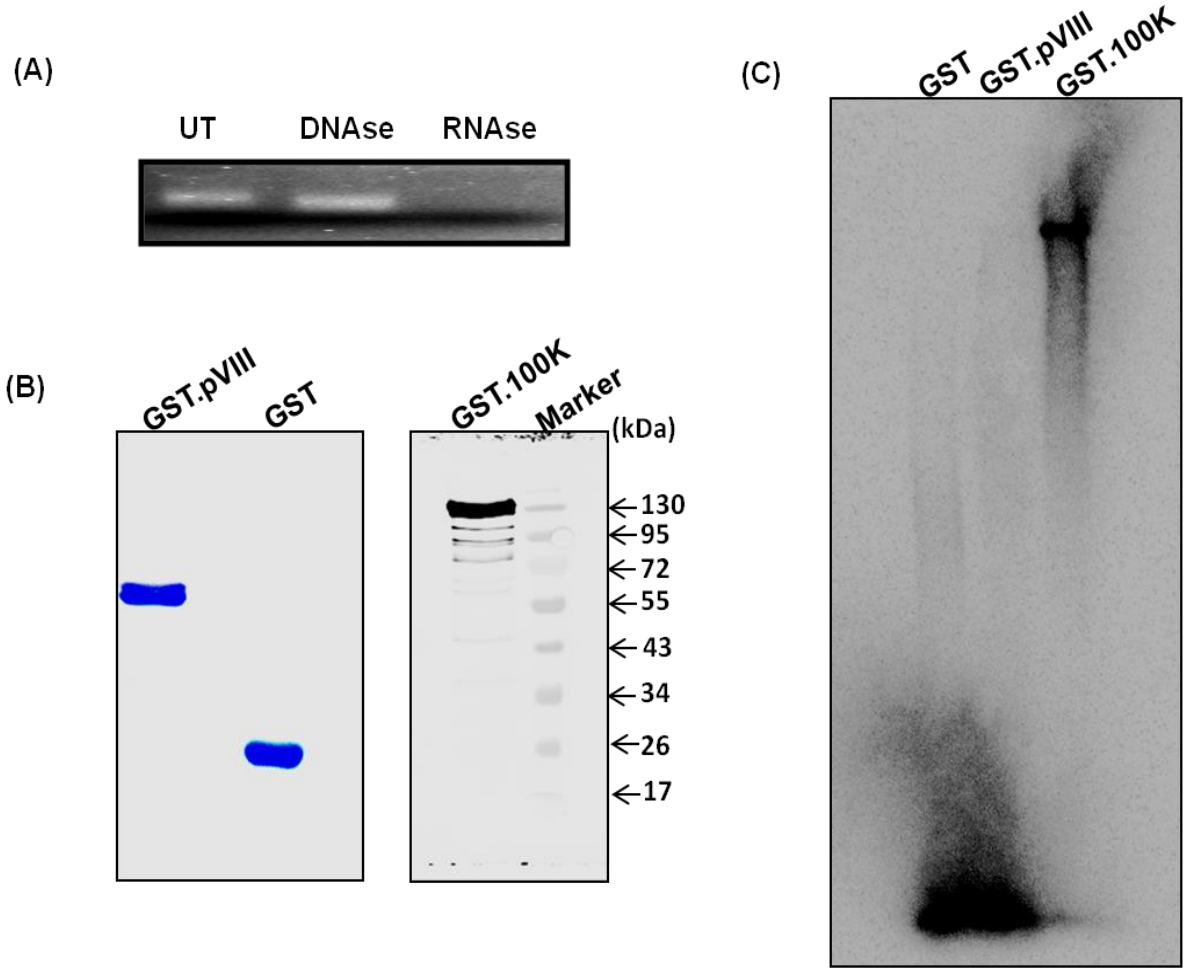


Figure 5.3.10 Electrophoretic mobility shift assay. (A) Oligo dT column purified cellular polyA RNA undigested (UN) or digested with either DNase or RNAse enzymes was analyzed by agarose gel electrophoresis. **(B)** [^{32}P] (UTP) Labeled (10000 cpm) polyA containing cellular RNA purified from the cytoplasmic fractions of MDBK cells was incubated either with 500ng of GST alone , BAdV-3 pVIII fused to GST (GST.pVIII) or BAdV-3 100K fused to GST (GST.100K) fusion protein for 30min, separated by 4% acrylamide native gel and detected by autoradiography.

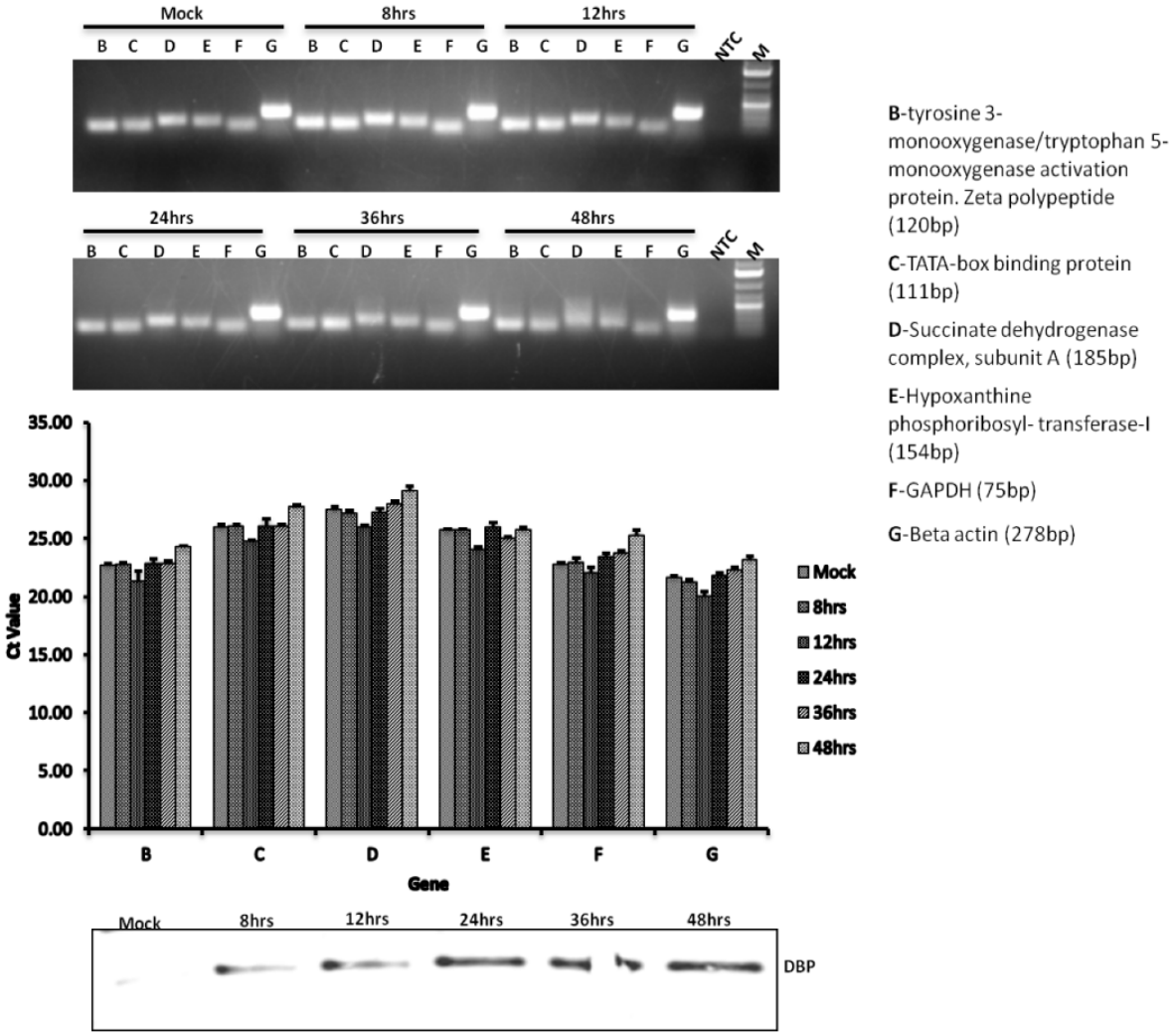


Figure 5.3.11 RT-PCR. MDBK cells were infected with 5 MOI of BAdV-3. At indicated times post infection cytoplasmic RNA was purified and cDNA synthesized and RT-PCR performed using specific primers targeting the indicated bovine housekeeping genes as described in the materials and methods. Western blot was performed using anti-BAdV-3 DBP (DNA binding protein) to confirm productive infection. Error bars indicate S.E of means for three separate experiments.

and alter the normal function of DDX3 in cells (Schroder, 2011). Vaccinia virus K7 protein inhibits interferon production by interacting with DDX3, which is involved in the activation of interferon regulatory factor (IRF) by directly binding with TBK1 and IKKϵ (Schroder et al., 2008). The core protein of the hepatitis C virus abrogates interferon production by associating with DDX3 (Breiman et al., 2005). Similarly, the hepatitis B virus polymerase inhibits IRF signaling via its interaction with DDX3 (Wang and Ryu, 2010; Yu et al., 2010). Besides, Rev protein of HIV exports intron containing viral transcripts from the nucleus into the cytoplasm by binding with DDX3 through the CRM1 dependent export pathway (Yedavalli et al., 2004).

The exact role of DDX3 in cap dependent translation is not clear. While some reports indicate that DDX3 acts as a general translation initiation factor (Lee et al., 2008; Geissler et al., 2012), another report indicate that DDX3 promotes the translation of a subset of selected mRNAs with structured mRNAs at their 5' end (Soto-Rifo et al., 2012). In addition, Ded1 (the yeast homologue of DDX3) have been shown to promote general translation initiation by enhancing the formation and resolution of an eIF4F-mRNA complex (Hilliker et al., 2011) However, Shih et al., 2008 reported DDX3 as a translation repressor. Here, we provide evidence for the role of DDX3 in cap dependent mRNA translation and report that interaction of DDX3 with BAdV-3 pVIII alters the translation of cellular mRNAs by depleting eukaryotic initiation factors from the cap binding complex. This may selectively allow translation of BAdV-3 specific mRNAs at late times post infection leading to efficient replication of BAdV-3.

Like human adenovirus-5 (Huang and Schneider, 1991; Yueh and Schneider, 1996), global cellular protein synthesis was inhibited at late times post BAdV-3 infection, which appears to be linked with the activity of adenovirus late protein(s). Since DDX3 acts to promote cap dependent translation initiation (Geissler et al., 2012; Soto-Rifo et al., 2012), the interaction of DDX3 with

pVIII may interfere with translation of cap dependent mRNAs. Several observations support this speculation. First, the inhibition of cellular protein synthesis observed at late times post infection correlates with the expression of pVIII in BAdV-3 infected cells. Secondly, expression of pVIII in transfected cells alters cellular protein synthesis in dose and time dependent manner. Thirdly, *in-vitro* translation of only capped luciferase mRNAs is inhibited in the presence of purified GST-VIII, but not in the presence of GST alone. Fourthly, pVIII does not alter the mRNA stability or transport to the cytoplasm. Finally, translation of only capped renilla luciferase mRNA is inhibited in transfected cells expressing EY.pVIII but not in transfected cells expressing EYFP.

Translation of eukaryotic mRNAs involves the binding of mRNA cap (5'm^7GpppN) structure to cap binding protein complex (eIF-4F), which include eIF4E, eIF4A and eIF4G (Jackson et al., 2010) proteins. However, alterations in one or more eIFs may impair the cap dependent translation of cellular mRNAs (Hinnebusch and Lorsch, 2012; Jivotovskaya et al., 2006). A number of viruses inhibit host protein synthesis (Bushell and Sarnow, 2002; Schneider and Mohr, 2003) by modifying the activity of eukaryotic initiation factors, which are required for bringing ribosomes to capped mRNAs (Feigenblum and Schneider, 1993), by sequestration of PABP (Ilkow et al., 2008), cleaving eIF4G and/or dephosphorylation of 4E-BP1 (You et al., 1999), or decreasing the activity of eIF4E (Burgui et al., 2007; Connor and Lyles, 2002) by direct binding of viral protein(s) with eIFs.

Previously it was reported that direct binding of adenovirus 100K to eIF4G induces under phosphorylation of eIF4E by displacing Mnk1 leading to the inhibition of cap-dependent translation (Cuesta and Schneider, 2004; Cuesta and Schneider, 2000). A different strategy appears to be adopted by BAdV-3 pVIII protein to alter the host protein synthesis. Although eIFs

are not degraded in BAdV-3 infected cells, the availability of eIFs for binding to mRNA cap (5'm7GpppN) structure appears to be depleted in BAdV-3 infected or pVIII transfected cells. Absence of binding of pVIII to eIFs in DDX3 depleted cells suggest pVIII does not appear to interact directly with eIFs. Since DDX3 interacts with translation initiation factors including eIF3, eIF4E and PABP (Lee et al., 2008; Lai et al., 2008; Shih et al., 2012; Shih et al., 2008), the interaction of pVIII with DDX3 depletes DDX3 along with associated eIFs from cap binding protein complex and thus inhibiting cellular protein synthesis in BAdV-3 infected cells. Recent reports suggest that DDX3 promotes cap dependent translation initiation by directly interacting with eukaryotic initiation factor eIF3 in an RNA independent manner (Yedavalli et al., 2004; Lai et al., 2008; Lee et al., 2008; Geissler et al., 2012). The eIF3 is a multi-subunit mammalian initiation factor, which binds to 40S ribosomal subunit preventing premature joining of 40S and 60S ribosome subunits, and also interacts with eIF4G to help to recruit m^7G capped mRNAs promoting the formation of 43S pre-initiation complex (Jackson et al., 2010). It is possible that interaction of pVIII with DDX3 sequesters both DDX3 and eIF3 modulating the efficient formation of 43S preinitiation complexes thus affecting the translation of capped mRNAs. Our results demonstrate that addition of pVIII cause significant reduction in the amount of available eIF3 and DDX3, thus limiting the translation of capped mRNAs *in-vitro* or *in-vivo*. Similarly, m^7GTP Cap analog captured significantly reduced level of DDX3 and eIF3 from extracts of BAdV-3 infected or pVIII expressing plasmid DNA transfected cells. However, such preinitiation complexes are loaded on adenovirus late mRNAs (Yueh and Schneider, 1996). It is possible that interaction of BAdV-3 pVIII with DDX3 does not affect the formation of 43S pre initiation complex, but impairs the ability of eIF3 to interact with eIF4G thus abolishing the association of 43S complex with capped mRNAs. Vpg protein of Norwalk virus interacts with

eIF3 and inhibits translation of capped mRNAs possibly by interfering with interaction of eIF4G with eIF3 (Daughenbaugh et al., 2003). Moreover, no pVIII was detected in m7GTP resins which rules out the possibility of the competitive binding of pVIII with m7GTP cap.

Earlier reports suggested that binding of adenovirus late protein 100K to eIF4G may displace kinase Mnk1, thus affecting the phosphorylation of translation eukaryotic initiation factor eIF4E (Cuesta and Schneider, 2000; Cuesta and Schneider, 2004). Under-phosphorylation of eukaryotic initiation factor eIF4E late (~40hrs) in adenovirus infected cells is thought to destabilize the interaction of cap structure of cellular mRNAs with eIF4F in 43S initiation complex (Huang and Schneider, 1991; Yueh and Schneider, 1996). Although reduced phosphorylation of eIF4E is usually associated with inhibition of cellular capped mRNAs translation (Huang and Schneider, 1991), the specific mechanism is still not clear.

Binding of adenovirus 100K to eIF4G does not affect the binding of eukaryotic initiation factors eIF4E, eIF4A and PABP to cap initiation complex eIF4F *in-vivo* (Cuesta and Schneider, 2000). Similarly, interaction of DDX3 with pVIII in transfected or virus infected cells did not affect the binding of eIF4G with 7-Methyl Guanosine Cap structure analog. In contrast, interaction of DDX3 with pVIII significantly diminished the binding of not only eIF3 but also eIF4E or PABP to 7 -Methyl Guanosine Cap structure analog. While PABP mediates binding of eIF4E and eIF4A to capped and polyadenylated mRNAs by interacting with eIF4G (Brook et al., 2012), eIF4E is the least abundant protein of translation initiation complex, thus it controls the rate of formation of eIF4F complex (Jackson et al., 2010). It is possible that availability of diminished amount of PABP and /or eIF4E affect the assembly of eIF4F complex in infected cells. Indeed, earlier reports suggest that limited availability of eIF4E (Feigenblum and

Schneider, 1993: Edgil et al., 2006) or PABP (Piron et al., 1998) in cap initiation complex, eIF4F inhibit translation of capped mRNAs.

6. TRANSITION FROM SECTION-5 TO SECTION-7

So far, I described the characterization of pVIII and its role in modulating the cap dependent cellular mRNA translation by interacting with DDX3. In the next section, I explored the role of pVIII-DDX3 interaction in enhancing the translation of BAdV-3 late mRNAs containing tripartite leader sequences (TPL) at 5'end of mRNAs.

7. DDX3 AND pVIII INTERACT WITH THE TRIPARTITE LEARDER (TPL) OF BAdV-3 AND ENHANCE TRANSLATION OF LATE VIRAL mRNAs AND PRODUCTION OF PROGENY VIRIONS

7.1. INTRODUCTION

Adenoviruses are non enveloped icosahedral DNA viruses that infect a wide variety of hosts. In infected cells, members of *Mastadenovirus* genus produce virus specific transcripts in four distinct phases including pre-early, early, intermediate and late. Although early gene expression does not require viral DNA replication, the late gene expression is dependent on initiation of DNA replication. Late transcription is controlled by the major late promoter (MLP) and produces transcripts that primarily encode structural proteins. All late transcripts contain an identical 5' non-coding segment called the tripartite leader (TPL) and are divided into different classes depending on the usage of polyadenylation signals. The TPL enhances the translation of mRNAs in infected and transfected cells. The TPL of BAdV-3 is 205 nucleotides long and is derived from three exons (Reddy et al., 1998). Bovine adenovirus (BAdV)-3 is being characterized at the molecular level (Baxi et al., 2000; Kulshreshtha et al., 2004; Reddy et al., 1998; Reddy et al., 1999a; Wu and Tikoo, 2004; Xing and Tikoo, 2007; Zakhartchouk et al., 1998; Zakhartchouk et al., 1999; Zhou and Tikoo, 2001; Zhou et al., 2001) with the aim of understanding the virus cell interactions. Like other members of *Mastadenoviruses*, the late transcriptional units of BAdV-3 genome are divided in to seven classes L1-L7 (Reddy et al., 1998). The late transcript region-6 (L6) of BAdV-3 encodes 100K, 33K and pVIII proteins (Reddy et al., 1998). Earlier, we demonstrated that BAdV-3 pVIII, a protein of 216 amino acids long containing two recognizable adenovirus protease specific cleavage sites (^{108}AGG↓G and

^{143}LGGG↓S) (Reddy et al., 1998) and predominantly localizes to the nucleus by interacting with importin(s) (Ayalew et al., 2014).

Viruses depend on host cell processes and factors for productive replication. Studying cellular targets of viral proteins often gives a novel insight about the functional importance of the protein and in designing efficient therapeutic and viral manipulation strategies. We have previously identified DDX3, member of the family of DEAD (Asp-Glu-Ala-Asp) box RNA helicases (Ditton et al., 2004; Gross et al., 2007; Tarn and Chang, 2009), as a cellular target of pVIII. We have also demonstrated that pVIII inhibits the translation of cellular capped mRNAs by depleting eIF3, eIF4E and PABP from the cap-binding complex without affecting levels and integrity of cytoplasmic mRNA (Chapter 5).

Recent reports have suggested the involvement of DDX3 in the replication of vaccinia virus (Schroder et al., 2008), hepatitis B virus (Wang and Ryu, 2010; Yu et al., 2010), hepatitis C virus (Ariumi et al., 2007; Oshiumi et al., 2010; Owsianka et al., 1999; You et al., 1999) and human immunodeficiency virus (HIV)-1 (Liu et al., 2011; Yedavalli et al., 2004). Here, we report a decrease in formation of functional 80S ribosomes in pVIII expressing plasmid transfected cells. Alternatively, DDX3 and pVIII interact with the TPL of BAdV-3 and enhance the translation of RNAs containing TPL at their 5' ends. Moreover, we demonstrate that DDX3 is required for efficient production of progeny virus at late times post-infection.

7.2. MATERIALS AND METHODS

7.2.1. Cells and Viruses

Madin-Darby bovine kidney (MDBK) cells, HeLa cells and 293T cells were grown in minimum essential medium (MEM; Sigma Aldrich) containing 5% FBS (SeraCare Life

Sciences, Inc.). VIDO R2 cells (Reddy et al., 1999) were grown in MEM containing 10% FBS. Wild-type BAdV-3 was propagated in MDBK cells (20). Cotton rat lung (CRL) cells were grown in MEM containing 2% FBS (Du et al., 2010).

7.2.2. Antibodies

Production of anti-DNA binding protein (Zhou et al., 2001) and anti-fiber (Wu and Tikoo, 2004) antibodies have been described earlier. Anti-hexon antibodies generated against BAdV-3 hexon recognize a protein of 98 kDa in BAdV-3 infected cells (Kulshreshtha et al., 2004). Anti-pVIII recognizes a protein of 24kDa in BAdV-3 infected cells (Ayalew et al., 2014). Anti-DDX3 serum (Santa Cruz Biotechnology, Inc, USA), goat anti-rabbit IRDye® 680 and Goat anti-mouse IRDye680 (Mandel Scientific Company, Inc, Canada) were purchased.

7.2.3. Construction of plasmid DNAs

The construction of plasmids pLV-DDX3i#7, pMD2.G and pcMV-ΔR8.91 (a gift from Dr. Yasuo Ariumi, Okayama University, Japan) have been described earlier (Ariumi et al., 2007). Plasmid pC.VIII (Gaba and Tikoo, unpublished) contains BAdV-3 pVIII gene cloned in plasmid pcDNA3 (Invitrogen).

Plasmid *pGL-bMLP*: the major late promoter (MLP) of BAdV-3 with the tripartite leader was cloned into plasmid pGL upstream of a firefly luciferase reporter gene. Initially, the MLP was amplified by PCR using " GCCGCGGGGAGTGCCTC TGTCTTCTAAATAGAGGATG" as a forward primer and "AAGCTTGGCGCGCCATCA TATGGTGCACACCCACTAGCAAT" as a reverse primer and plasmid PUC304A+ (Du and Tikoo, 2010) DNA as a template. The PCR

product was digested with *Sac*II and *Hind*III restriction enzymes and ligated into *Sac*II and *Hind*III digested plasmid pGL (Promega) creating plasmid pGL-bMLP.

To clone the spliced form of TPL downstream of the MLP, a 217bp DNA fragment was amplified by RT PCR using primers using primers (forward: 5'-ATCATATGTGACGGTGCTTCCGC GTGGCATCC-3'; reverse: 5'-GGCGCGCCCTTGTGACTGCGACTGGT TAGAAGT-3') and RNA purified from MDBK cells 36 hr post infection with BAdV-3. The RT-PCR amplified 217bp fragement was digested with *Asc*I and *Nde*I enzymes and cloned into *Asc*I and *Nde*I digested pGL-bMLP creating plasmid pGL-bMLP-TPL.

Plasmid *pGE.VIII.* A 666bp *Hind* III (blunt end repaired with T4 polymerase) - *Xho*I fragment of plasmid pR.pVIII was ligated to *Aat*II (blunt end repaired with T4 polymerase) - *Xho*I digested plasmid pGEX-5X-1 (GE Healthcare) creating plasmid pGE.pVIII.

Plasmid *pGST.DDX3.* A 2062bp *Bam*HI (blunt end repaired with T4 polymerase)-NotI fragment was isolated from pHA.DX3 and ligated to *Sal*I (blunt end repaired with T4 polymerase) -*Not*I digested plasmid pGEX-5X-2 (GE Healthcare) creating plasmid pGST.DDX3.

7.2.4. Recombinant protein expression and protein purification

The recombinant GST-fusion proteins were expressed in *Escherichia coli* BL21 cells as described (Zhou and Tikoo, 2001). Glutathione S-transferase (GST), GST-pVIII and GST-DDX3 fusion proteins were purified using GST beads (GE Healthcare) as per the instructions of the manufacturers. The purified proteins were dialyzed using Slide-A-Lyzer dialysis cassette

(Thermo Scientific). The concentrations of the proteins were measured by Bradford assay (Bio-Rad) using Ultrospec® 3000 spectrophotometer (Pharmacia Biotech).

7.2.5. Production of lentivirus

Lentiviruses expressing DDX3 shRNA or scrambled shRNA were produced as described (Al Yacoub et al., 2007). About 5.8 x 10^6 293T cells were seeded in 10 cm^2 petri-dish in 6 ml complete DMEM containing 10% FBS. After overnight incubation, the cells were transfected with three plasmids pLV-DDX3i#7 (36µg; transfer vector), pMD2.G (18µg; envelope vector) and pcMV-ΔR8.91 (18µg; packaging construct) using calcium phosphate method as described (Du et al., 2010). After 5hr of incubation, the media was removed and replaced with 8ml of advanced DMEM containing 2% FBS, 0.01mM cholesterol, 0.01mM lecithin and 1x chemically defined lipid concentrate (Invitrogen). After 36hrs of incubation, the supernatant was collected, centrifuged at 1500rpm and passed through 0.45µm filter. Finally, the virus titer was quantified using an HIV-1 p24 antigen capture kit (Clontech) before storing at 70^{o}C.

7.2.6. Lentivirus transduction

VIDO R2 cells (Reddy et al., 1998) grown in 12 well plate (1x10^5/well) were treated with 8µg/ml of polybrene for 15 min and transduced with lentivirus. After overnight incubation at 37^{o}C, the media was replaced with selection media containing DMEM, 2% serum and 10µg/ml of puromycin. The media was changed every 3-4 days until appearance of puromycin resistant cell clones. The DDX3 gene silencing in selected puromycin resistant clones was verified by Western blot using DDX3 specific antibodies.

7.2.7. Tetrazolium/Formazan (XTT) assay

VIDO R2 (DDX3 positive) or VIDO R2kd (DDX3 knockdown) cells (1.5 x 10^4/well) were seeded in 96 well plates. After overnight incubation, XTT (Sigma Aldrich) was reconstituted with MEM without FBS at a concentration of 1mg/ml and filter sterilized with a 0.2µm syringe filter. Fifty µl of 5mM of Phenazine methosulfate (PMS) (Sigma Aldrich) was added to 5ml of XTT solution (1mg/ml). A volume of the reconstituted XTT solution equal to 20% of the culture medium to be tested was added to the cells. Finally, the cultures were incubated for 4hrs at 37^{o}C and the absorbance was measured at 450nm in an ELISA multi-well plate reader.

7.2.8. Viral replication in DDX3 silenced bovine cells

The indicated cells grown in 6 well plates were infected with wild-type BAdV-3 at an MOI of 5. At different times post-infection, the cells were lysed in the medium by freeze-thawing five times. The virus titer was determined by $TCID_{50}$ in MDBK cells.

7.2.9. Virus mRNA and protein synthesis in DDX3 silenced bovine cells

To analyze the viral mRNA in the cytoplasm of BAdV-3 infected cells, the cells seeded in 6 well plates were infected with wild-type BAdV-3 at an MOI of 5. At 48 hr post-infection, the cytoplasmic RNA was extracted by SurePrep^{TM} Nuclear or Cytoplasmic RNA purification kit (Fisher Bioreagents) as per the company's protocol. The concentration of the RNAs was measured by NanoDrop and cDNA was synthesized using equal amounts of RNA from DDX3 positive VIDO R2 or DDX3 knocked down VIDOR2kd cells by using Superscript II reverse transcriptase (Invitrogen) and random hexamers. Real-time PCR reactions were performed by

iCycler iQ^{TM} real-time PCR detection system (Bio-Rad) and the amplifications were performed using the SYBR green PCR master mix (Bio-Rad) using specific primers (Table 7.2.1). The RT-qPCR products were separated by agarose gel and the data was analyzed by Microsoft Office Excel 2007.

To determine the level of viral proteins in BAdV-3 infected cells, the cells seeded in 6 well plates were infected with wild-type BAdV-3 at an MOI of 5. At the indicated times post infection, proteins from the lysates of the infected cells were separated by 10% SDS-PAGE, transferred to a nitro-cellulose membrane and probed by Western blot using protein specific antibodies followed by goat anti-rabbit IRDye®680 as secondary antibody. β-actin was used as a loading control. Finally, the Western blots were scanned by using Odyssey® infrared imaging system (LI-COR). The intensity of the bands was measured by Odyssey software**.**

7.2.10. Luciferase assay

DDX3 Knockdown VIDO R2kd or wild type 293T cells were co-transfected with plasmid pEYFP.VIII + pGL-bMLP (containing only BAdV-3 MLP) or pEYFP.VIII + pGL-MLP-TPL (containing BAdV-3 MLP and TPL). At 36hrs post transfection, the cells were lysed and the firefly luciferase activity was measured in a luminometer by using a luciferase assay kit (Promega) as per the manufacturer's instructions. The experiment was performed two times in triplicates.

7.2.11. RNA- Electrophoretic mobility shift assay (EMSA)

The EMSA was carried out following a modification of the protocol by Yakhnin et al, 2012. About 10,000 cpm ^{32}P (UTP) radiolabeled RNA probe of *in-vitro* transcribed firefly

Table 7.2.1. List of primers

Primer name	Primer sequence
Hexon-F	5’-ATGGTCCTACTGCACATCGCCGTTC
Hexon-R	5’-CTGCCTAAACTGCGTATTGTTAGGG
Fiber-F	5’-ATGAAGAGAAGTGTGCCCCAGGAC
Fiber-R	5’-CACCTTGTCATCGGTTACCGTGAG
pIX-F	5’-ATGGCCGAGGAAGGGCGCATTTATGTG
pIX-R	5’- GTTAACTTGGCCGCCCACAAGGTTAGCCTGCAC
pVIII-F	5’-CGGCCGCAGGTGCCAGTCAAGATTACTC
pVIII-R	5’-CCTCTACAAACTGCCTGGATCCTATGC
DBP-F	5’-ATGAATCGCAGCGGTGACTTAACGCGAGCC
DBP-R	5’-GTCGTCCCCAGGCCTGCGACTTTTAAC
100k-F	5’-ATCAGCGGCCAGAGAACGACACTGAGC
100k-R	5’-TCTTGGGTGGTACCTTGGGACAAAAGAG
pV-F	5’-GGCCTCCTCTCGGTTGATTAAAGAAG
pV-R	5’-ACTACGTAAGTCGGTTCCGGCACGGCCACAGC
IVa2-F	5’-TGCTGGATGGAGATGTACTAGACCAC
IVa2-R	5’-TGCTGCTTTGAGGAACCAGAGTTCCTGTG

luciferase RNA with different 5'UTR (i.e. TPL, IRES or Cap) was allowed to bind with 500ng of GST, GST.DDX3 or GST.VIII fusion protein plus 10µg yeast tRNA (Ambion) at room temperature for 30min. Afterwards, the reaction mixture was run in a 4% acrylamide native gel for 4-6 hr at 150 V and the gel was dried, exposed onto a phosphor screen (Kodak) and scanned on a Molecular Imager FX using Quantity One software (Bio-Rad).

7.2.12. Polysome profile analysis, RNA and protein purification

293T cells grown in 100mm dish were transfected with plasmid pcDNA3 or pcVIII DNAs. At 40hrs post transfection, cyclohexamide was added to the medium with a final concentration of 50 µg/ml and the cells were further incubated for 30 min at 37^{o}C. Subsequently, the cells were washed twice with cyclohexamide containing PBS and collected in 2ml tubes. Then, polysome extraction buffer was added and the cells were incubated on ice for 10 min andcentrifuged to pellet the nuclei. The supernatants were then collected and OD values were measured at 260nm on a NanoDrop. A 5%-50% linear sucrose gradient was prepared in ultracentrifuge tubes and equal OD units from pcDNA3 or pcpVIII transfected cells were applied. The gradient was centrifuged at 36000 rpm for 2 hr and 30 min at 4^{o}C. From each tube 21 fractions of ~550µl were collected and absorbance was measured at 260nm and plotted on graph using Microsoft Excel. RNA and proteins were extracted from each fraction using Trizol reagent following the manufacturer's instructions. RNA was analysed by Agilent 2100 Bioanalyser instrument. Proteins from 1-16 fractions were separated by 12% SDS-PAGE gel, transferred onto nitrocellulose membrane and probed with anti DDX3 and anti-pVIII antibodies.

7.3.RESULTS

7.3.1. shRNA mediated knockdown of DDX3

A lentivirus vector mediated RNA interference was used to knock down DDX3 expression in bovine cells. Initially, we attempted to knockdown DDX3 expression in BAdV-3 permissive (MDBK, CRL) cells (Figure 7.3.1A) or non-permissive (HeLa) cells (Figure 7.3.1B). Although, we were able to knockdown DDX3 expression in HeLa cells (Figure 7.3.1B; 2), our repeated attempts to knockdown DDX3 expression in MDBK or CRL (Figure 7.3.1A) cells proved futile. Earlier studies have suggested that inability to efficiently knockdown DDX3 expression could be due to high level of expression or stability of DDX3 in these cells (Schroder et al., 2008; Yedavalli et al., 2004).

In contrast, we were able to knockdown DDX3 expression in BAdV-3 permissive VIDO R2 cells (Figure 7.3.1C). Briefly, lentiviral particles containing DDX3 shRNA (Ariumi et al., 2007) were used to transduce VIDO R2 cells. After isolating puromycin resistant clones, the knockdown of DDX3 expression was determined by Western blot using anti-DDX3 serum. As seen in Figure 7.3.1C, one (clone 5) of the six puromycin resistant clones was viable (panel D) and showed significant reduction (96%) in the expression of DDX3 (panel C). This clone was designated as VIDO R2kd. Similarly, puromycin resistant clone obtained by transducing VIDO R2 cells with lentivirus containing scrambled shRNA was designated as VIDO R2s.

7.3.2. Virus titer in VIDO R2kd cells

To determine the biological function of DDX3 during BAdV-3 infection, we determined the titer of BAdV-3 in VIDO R2kd cells. The cells were infected with wild type BAdV-3 at an MOI of 5. At indicated times post infection, the infected cells were collected, freeze-thawed and

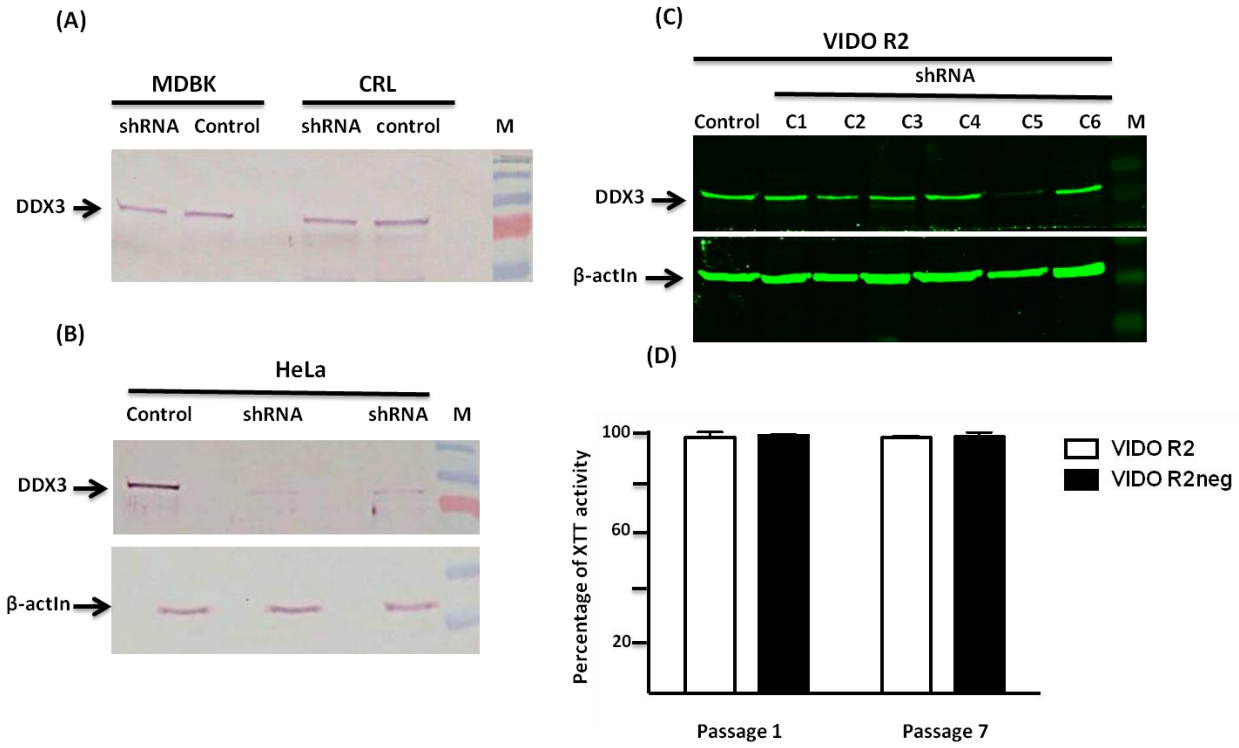


Figure 7.3.1. shRNA mediated knockdown of DDX3 in cells. MDBK and CRL (A), Hela (B) or VIDO R2 (C) cells were transduced with lentivirus vectors harboring DDX3 shRNA and selected with puromycin (10μg/ml). After 7 days, proteins from the lysates of puromycin resistant cells were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed in Western blot using anti-DDX3 MAb followed by IRDye800 conjugated anti-mouse secondary antibody (A, B) or anti-DDX3 serum followed by goat anti-rabbit IRDye® 680 as secondary antibody (C). β-actin is included as a loading control (B, C). XTT assay (D). The viability of VIDO R2 or VIDO R2kd (DDX3 knock down VIDO R2 clone 5) cells was determined by XTT assay as described in the materials & methods. Molecular weight markers are indicated in kilo Daltons (kDa).

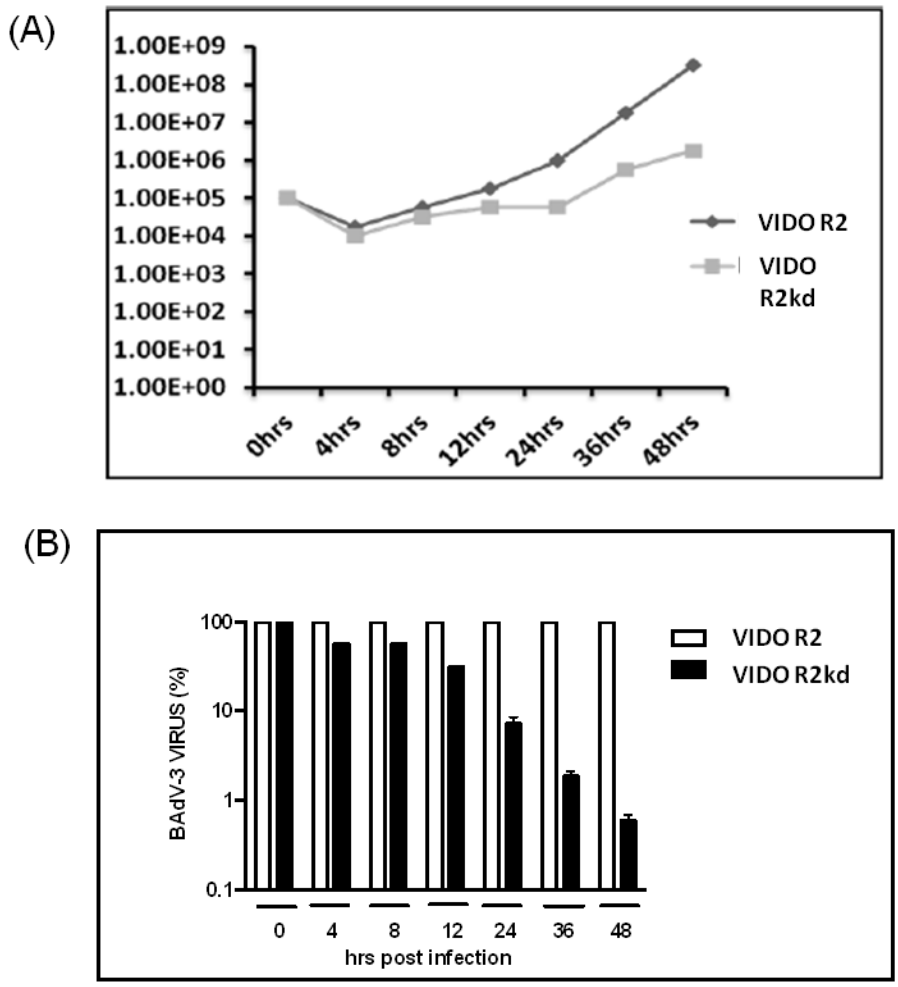


Figure 7.3.2. BAdV3 titer in DDX3 positive or DDX3 knockdown VIDO R2 cells. Near confluent monolayers of VIDO R2s or VIDO R2kd cells were infected with wild-type BAdV-3 at an MOI of 5. At indicated times post-infection, the infected cells were freeze-thawed and virus was titrated on MDBK cells as described in the text. (A) BAdV-3 titer in VIDO R2s and VIDO R2kd cells, (B) Bar diagram depicting BAdV-3 titer in VIDO R2kd cells relative to VIDO R2s.

the cell lysate was used to determine the BAdV-3 titer by $TCID_{50}$ assay. As seen in Figure 7.3.2, compared to VIDO R2s, shRNA knockdown of DDX3 in VIDO R2kd cells resulted in 3-fold, 18-fold, 63-fold and 210-fold reduction in virus titers at 12, 24, 36 and 48hrs post-infection (panels A,B), respectively.

7.3.3. BAdV-3 protein specific mRNA in VIDO R2kd cells

To determine if DDX3 knockdown affects the accumulation of late viral mRNAs in the cytoplasm of infected cells, RT-qPCR was performed using BAdV-3 gene specific primers (Table 7.2.1). As seen in Figure 7.3.3, there was no significant difference in the accumulation of early (DBP) or intermediate (pIX, pIVa2) protein specific viral mRNAs in the cytoplasm of BAdV-3 infected VIDO R2s or VIDO R2kd cells late (48hrs) in infection (panels A,B). Similarly, there was no significant difference in the accumulation of late protein (penton, hexon) specific viral mRNAs in the cytoplasm of BAdV-3 infected VIDO R2s or VIDO R2kd cells late in infection (48hrs).

7.3.4. BAdV-3 specific protein synthesis in VIDO R2kd cells

To determine the effect of DDX3 knockdown on translation of BAdV-3 protein specific mRNAs, the level of BAdV-3 proteins produced in infected cells was determined by Western blot using protein specific antibodies, goat anti-rabbit IRDye®680 secondary antibody and Odyssey® infrared imaging system (LI-COR). As seen in Figure 7.3.4, there was no significant difference in the level of DBP (early protein) in BAdV-3 infected VIDO R2s or VIDO R2kdcells late (48 hrs) in infection (panels A, B). In contrast, there was significant difference in the level of fiber or

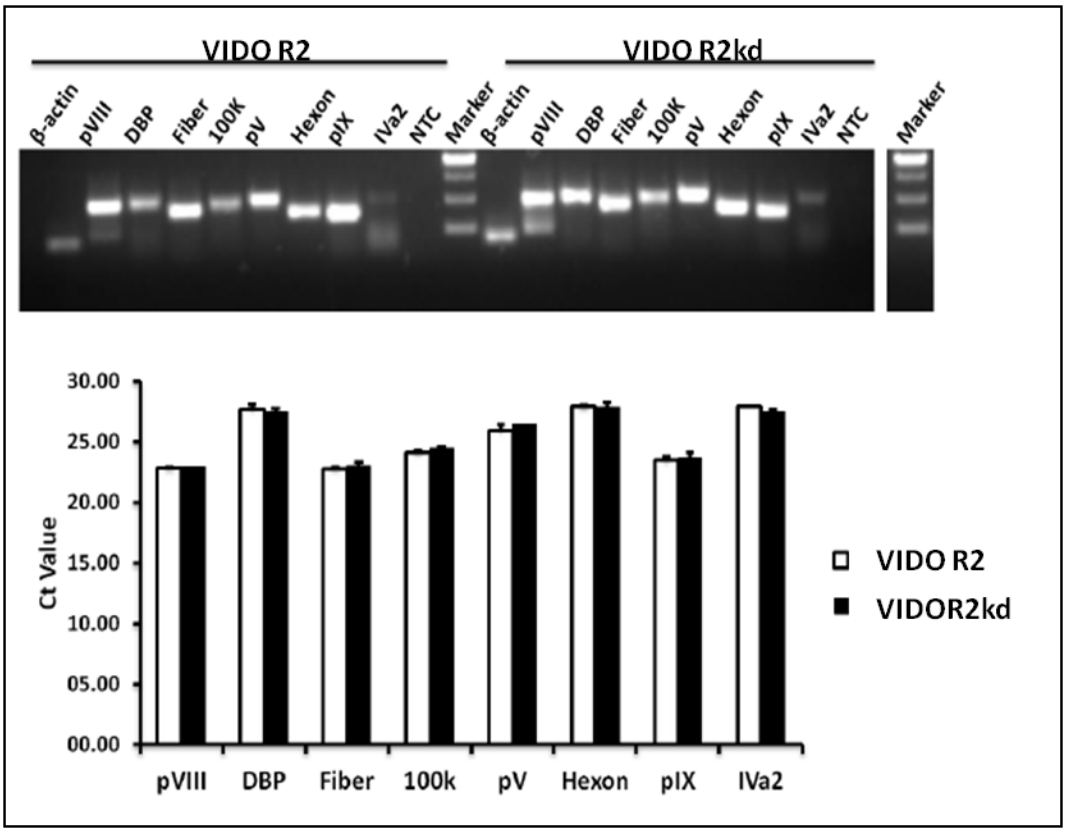


Figure 7.3.3. RT-PCR. (A) *Analysis of ethidium bromide-stained RT-PCR products.* Products of RT-PCR using DNAse-treated RNA isolated from BAdV-3 infected cells at 48hrs post-infection as a template were synthesized using primers pairs (Table 1). Size markers are shown in base pairs (bps). (B) Comparison of normalized Ct values of cytoplasmic mRNA levels of early, intermediate and late BAdV-3 gene products. β-actin was used as a house keeping gene.

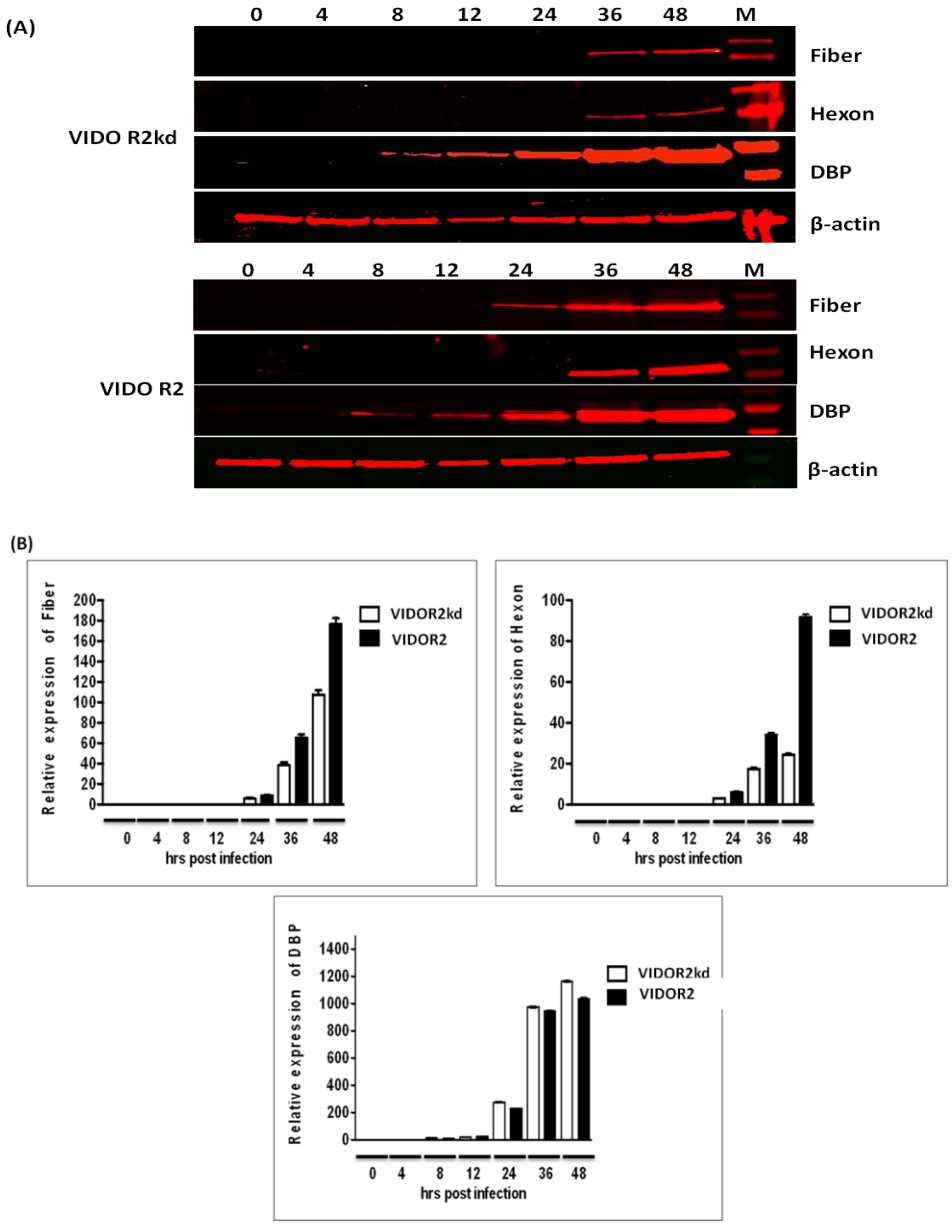


Figure 7.3.4. Western blot. (A) Proteins from the lysates of BAdV-3 infected cells collected at different times (0-48hrs) post-infection were separated by 10 % SDS-PAGE, transferred to nitrocellulose and probed in Western blot using protein specific antibodies followed by goat anti-rabbit IRDye® 680 as secondary antibody. β-actin is used as a loading control. (M) Protein markers. (B) The intensity of the protein bands in "A" was measured using Li-COR Odyssey software and normalized values were plotted using Microsoft Excel 2007.

hexon (both late proteins) proteins in BAdV-3 infected VIDO R2 or VIDO R2kd cells late (48hrs) in infection (panels A, B).

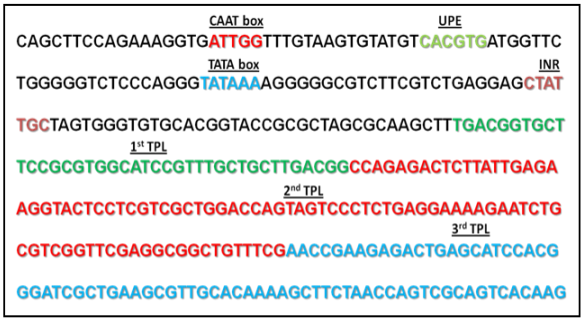
7.3.5. Luciferase assay

The late transcription units of BAdV-3 are transcribed from the Major Late Promoter (MLP). The mRNAs transcribed from the MLP contain an identical 5' non-coding segment called the tripartite leader (TPL) which is 205 nucleotides long in BAdV-3 and is derived from three exons (Figure 7.3.5A). To study the effect of DDX3 on the translation of late BAdV-3 mRNAs, the major late promoter of BAdV-3 with the tripartite leader was cloned into pGL vector (Addgene) upstream of a firefly luciferase reporter gene. Subsequently, the effect of DDX3 on the translation of late viral mRNA translation was studied by co-transfecting DDX3 knockdown (kd) or positive 293T cells (Figure 7.3.5B) with either plasmids pEYFP.VIII + pGL-bMLP (containing only BAdV-3 MLP) or plasmids pEYFP.VIII + pGL-MLP-TPL (containing BAdV-3 MLP and TPL). As seen in Figure 7.3.5C, the expression of the reporter gene is significantly higher in pGL-bMLP-TPL transfected DDX3 positive cells as compared to DDX3kdcells. No such difference was observed in the expression of the luciferase reporter gene in DDX3 positive or DDX3kd 293T cells co-transfected with plasmid pGL-bMLP + pEYFP.VIII (Figure 7.3.5C).

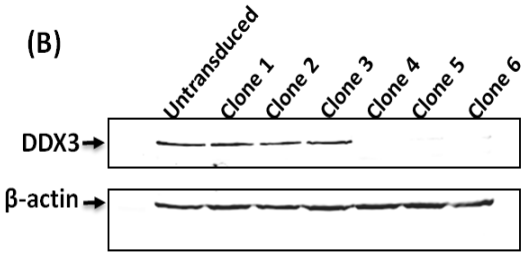
7.3.6. Interaction of pVIII and DDX3 with TPL of BAdV-3

TPL enhances the translation of viral mRNAs in infected and transfected cells (Dolph et al., 1988). Since the translation of a firefly reporter gene with 5' TPL under the control of MLP was enhanced in DDX3 positive 293T cells in the presence of pVIII, we investigated the

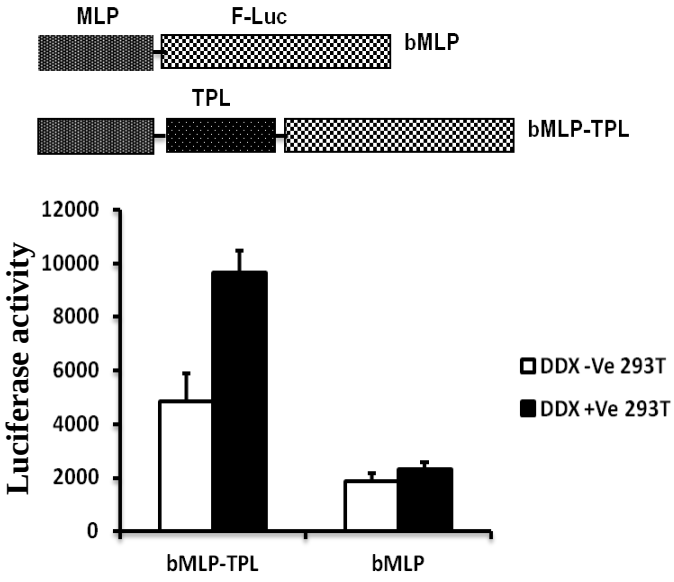
(A)


CAAT box
UPE
CAGCTTCCAGAAAGGTGATTGGTTTGTAAGTGTATGTCACGTGATGGTTC
TATA box
INR
TGGGGGTCTCCCAGGGTATAAAAGGGGGCGTCTTCGTCTGAGGAGCTAT
TGCTAGTGGGTGTGCACGGTACCGCGCTAGCGCAAGCTTTGACGGTGCT
1st TPL
TCCGCGTGGCATCCGTTTGCTGCTTGACGGCCAGAGACTCTTATTGAGA
2nd TPL
AGGTACTCCTCGTCGCTGGACCAGTAGTCCCTCTGAGGAAAAGAATCTG
3rd TPL
CGTCGGTTCGAGGCGGCTGTTTCGAACCGAAGAGACTGAGCATCCACG
GGATCGCTGAAGCGTTGCACAAAAGCTTCTAACCAGTCGCAGTCACAAG

(B)


Untransduced
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
DDX3
β-actin

(C)


MLP
F-Luc
bMLP
TPL
bMLP-TPL
Luciferase activity
12000
10000
8000
6000
4000
2000
0
bMLP-TPL
bMLP
DDX -Ve 293T
DDX +Ve 293T

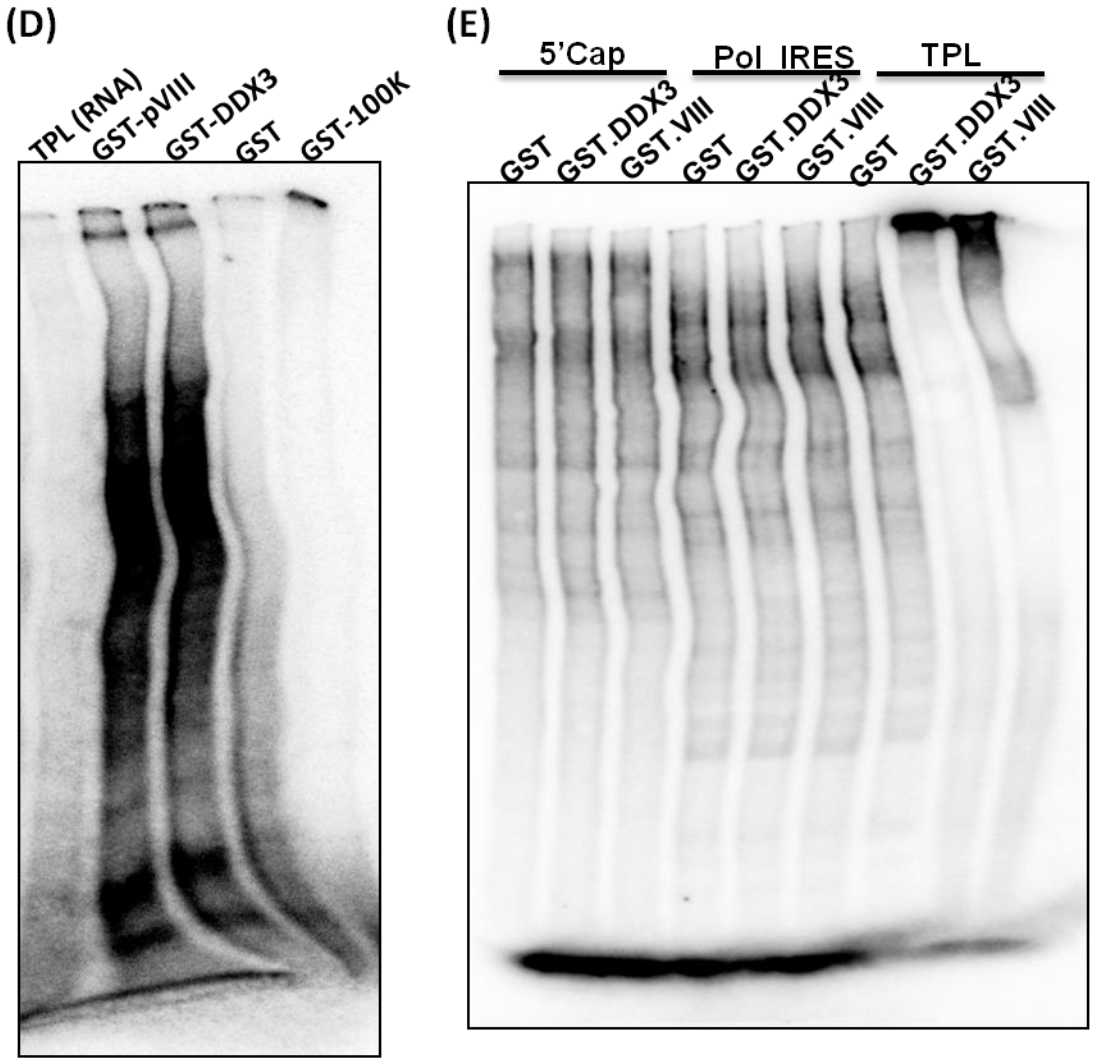
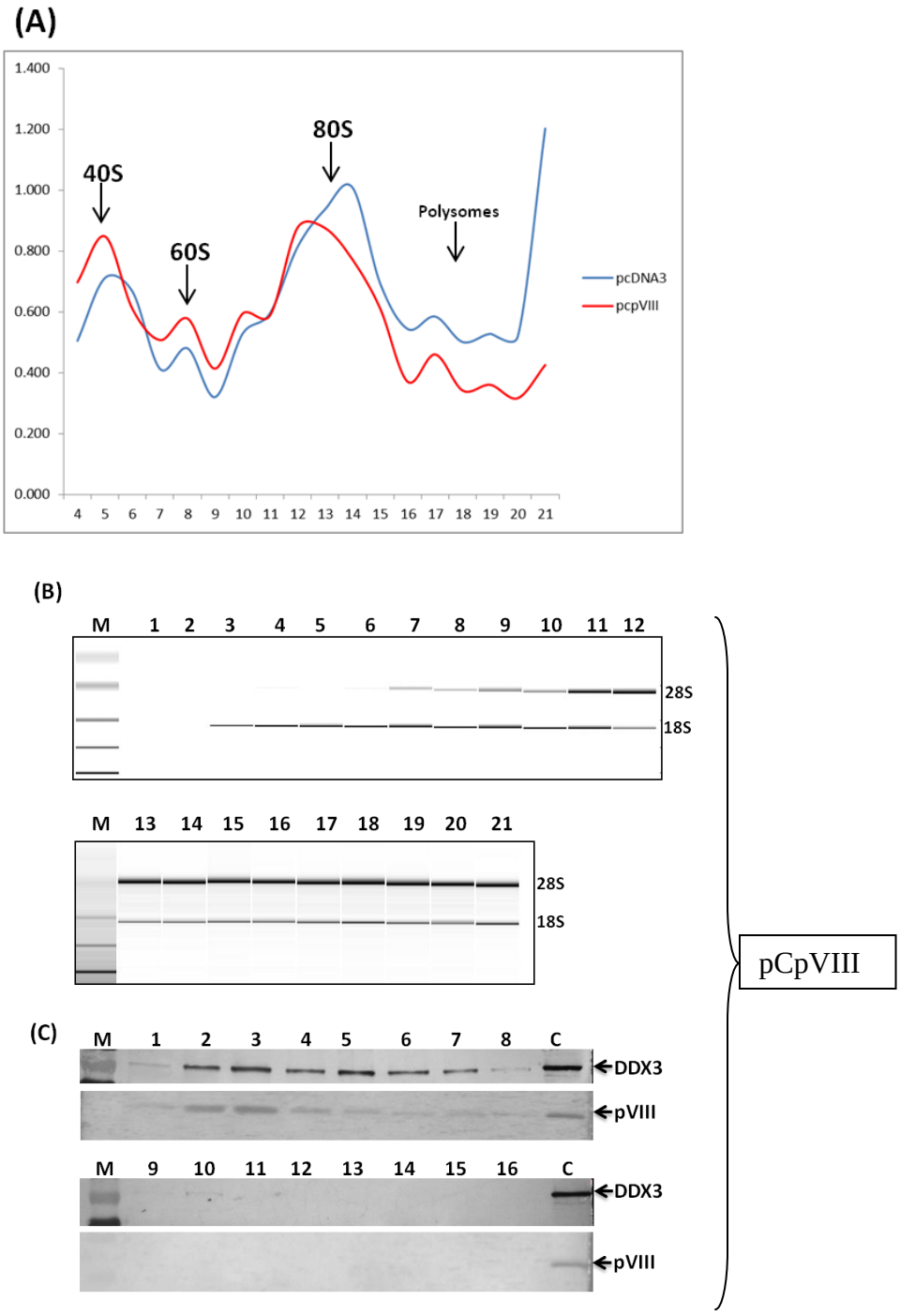

(D)
TPL (RNA)
GST-pVIII
GST-DDX3
GST
GST-100K
(E)
5'Cap
PoI IRES
TPL
GST
GST.DDX3
GST.VIII
GST
GST.DDX3
GST.VIII
GST
GST.DDX3
GST.VIII

Figure 7.3.5 (A) The nucleotide sequence of major late promoter (MLP) and tripartite leader (TPL) of BAdV-3. (B) shRNA mediated knockdown of DDX3 in 293T cells. 293T cells were transduced with lentivirus vectors containing DDX3 shRNA and selected with puromycin (10μg/ml). After 7 days, proteins from the lysates of puromycin resistant cells were separated by 10% SDS-PAGE, transferred to nitrocellulose and probed by Western blot using anti-DDX3 MAb followed by IRDye800 conjugated anti-mouse secondary antibody. β-actin is included as a loading control. **(C). Reporter gene expression**. DDX3 knockdown or positive 293T cells were co-transfected with either pEYFP.VIII and pGL-bMLP (containing only BAdV-3 MLP) or pGL-MLP-TPL (containing BAdV-3 MLP and TPL). The absolute expression of firefly luciferase was measured 36hrs post transfection in a luminometer. **(D & E) Electrophoretic mobility shift assay.** ^{32}P (UTP) radiolabeled RNA probe of *in-vitro* transcribed firefly luciferase RNA with different 5'UTR (i.e. TPL, IRES or 5'caps) was allowed to bind with 500ng of GST, GST-DDX3, GST-100K or GST.VIII protein at room temperature for 30min. Subsequently, the reaction mixture was run on a gel and autoradiographed

possibility of the interaction of pVIII and DDX3 with mRNAs of different 5' UTR. We initially tried to examine whether BAdV-3 TPL interacts with DDX3 and pVIII using GST-fused purified proteins. As expected (Cuesta and Schneider, 2004), GST.100K interacts with TPL (Figure7.3.5D). Similarly, both GST.pVIII and GST-DDX3 interacts with the TPL. No such interaction was observed between TPL and GST alone. Similarly, GST alone, GST.DDX3 or GST.pVIII fusion proteins did not show any interaction with 5' pol IRES or Cap containing RNA (Figure 7.3.5E)

7.3.7. Polysome profile analysis, RNA and protein purification and Western blot in pEY. VIII transfected cells

To monitor the efficiency of protein translation in 293T cells transfected with plasmid pcDNA3 or pc.VIII DNAs, polysome profile analysis was performed using sucrose gradient centrifugation technique. Based on 28S and 18S ribosomal RNA profiles, fractions 4 to 7, 7 to 10, 10 to 16 and 16 to 21 are identified as 40S, 60S, 80S and polysome fractions, respectively (Figure 7.3.6A, B). As shown in Figure 7.3.6, more free 40S and 60S and less 80S ribosome were observed in cells transfected with plasmid pcVIII DNA than in cells transfected with plasmid pcDNA3 DNA. In addition, the polysome peaks are lower in pc.VIII DNA than in pcDNA3 transfected cells (Figure 7.3.6A). To determine the presence of pVIII or DDX3 in different fractions, proteins were purified from the different fractions and probed by Western blot using either anti-pVIII or anti DDX3 antibody. As shown in Figure 7.3.6C, both DDX3 and pVIII were detected only in the fractions containing 40S ribosome and not in any of the other fractions.


(A)
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0.000
4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
40S
60S
80S
Polysomes
pcDNA3
pcpVIII
(B)
M 1 2 3 4 5 6 7 8 9 10 11 12
28S
18S
M 13 14 15 16 17 18 19 20 21
28S
18S
pCpVIII
(C)
M 1 2 3 4 5 6 7 8 C
DDX3
pVIII
M 9 10 11 12 13 14 15 16 C
DDX3
pVIII

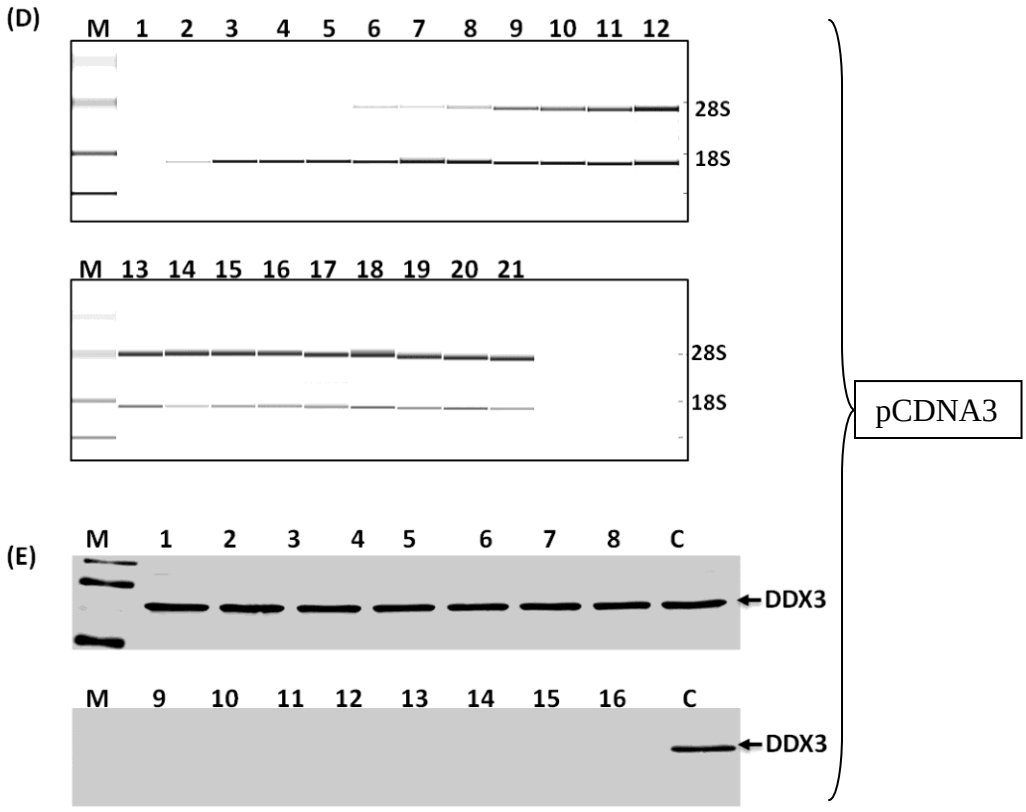


Figure 7.3.6. (A) Polysome profiling. 293T cells transfected with plasmid pc.VIII or pcDNA were collected 40 hr post transfection. The cells were lysed and the supernatants were added to a 5%-50% linear sucrose gradient and centrifuged at 36000rpm for 2hr 30min at 4^{o}C. 21 fractions of ~550µl were collected and absorbance was measured at 260nm and plotted on graph using Microsoft Excel. The graph shows 40S, 60S, 80S and polysome peaks. (B, D) **Analysis of 18S and 28S ribosmomal RNAs**. RNA was extracted from each fraction of pCpVIII (B) or pCDNA3 (D) transfected cell lysates using Trizol reagent as per the manufacturer's instructions. RNA was analysed by Agilent 2100 Bioanalyser instrument. (C, E). **Western blot.** Proteins extracted from fractions 1-16 of pCpVIII (C) or pCDNA3 (E) transfected cell lysates were run on a 12% SDS gel, transferred onto nitrocellulose membrane and probed with anti DDX3 or anti pVIII antibodies.

7.4. DISCUSSION

The interaction of viral proteins with cellular proteins is of vital importance in the regulation of virus replication, growth and survival. To properly understand their significance in the cells, identification of the interactions, understanding the extent to which they take place, and determining the consequences of such interactions are very essential (Phizicky et al., 1995). Earlier, we demonstrated that BAdV-3 pVIII is expressed as 24kDa protein in BAdV-3 infected or gene transfected cells, and is transported to the nucleus by interacting with importin(s) (Section 3). We also documented a novel interaction between BAdV-3 pVIII and a cellular protein DDX3 (Section 4). Here we report that DDX3 appears to be essential for efficient replication of BAdV-3. We also demonstrate that knockdown of DDX3 affects the translation of BAdV-3 late protein mRNAs without altering cellular protein translation in infected cells. Furthermore, both pVIII and DDX3 interacts with BAdV-3 TPL. With polysome profile analysis, we also show an overall decrease in cellular mRNA translation in pVIII expressing plasmid transfected cells.

The DEAD (Asp-Glu-Ala-Asp) box protein DDX3 is a member of the ATPase super family II helicases (Silverman et al., 2003), that is involved in various aspects of RNA metabolism (Kwong et al., 2005; Rocak and Linder, 2004). So far, viral proteins encoded by vaccinia virus, hepatitis B virus, hepatitis C virus and HIV have been shown to interact with DDX3 (Ariumi et al., 2007; Owsianka and Patel, 1999; Schroder et al., 2008; Wang and Ryu, 2010; Yedavalli et al., 2004; Yu et al., 2010; Lee et al., 2008), which appears to be required for efficient replication of these viruses. DDX3 is implicated in translation initiation (Lee et al., 2008; Parsyan et al., 2011) and may regulate cellular mRNA translation by interacting with

specific translation initiation factors eIF4E, eIF2α, PABP and eIF3 (Lai et al., 2008; Lee et al., 2008; Shih et al., 2008; Shih et al., 2011).

Knockdown of DDX3 resulted in significant reduction in the BAdV-3 yield, without altering the viability of the cells suggesting that DDX3 is required for efficient production of progeny virus. Interestingly, the reduction in virus production in VIDO R2kd (DDX3 knocked down) cells correlates with the degree of cellular protein synthesis inhibition in BAdV-3 infected VIDO R2s cells at late times (24-48hrs) post infection. It is possible that the reduction in the production of progeny BAdV-3 late in infection in VIDO R2kd cells could be due to reduced availability of BAdV-3 specific late mRNAs required for the production of progeny virions.

However, there appeared to be no difference in the amount of BAdV-3 specific mRNAs (early or late) in BAdV-3 infected VIDO R2s or VIDO R2kd cells at 48hrs post-infection. Alternatively, it is possible that the decrease in the production of progeny BAdV-3 late in the infection of VIDO R2kd cells could be due to inefficient translation of BAdV-3 late mRNAs. Although the level of early proteins appeared unaffected at late times post-infection of VIDO R2s or VIDO R2kd cells, the level of late proteins produced in VIDO R2kd cells was significantly reduced compared to the level of late proteins produced at late time post-infection of VIDO R2 cells. These results suggest that the absence of DDX3 in BAdV-3 infected VIDO R2kd cells abolishes the selective translation of BAdV-3 late mRNAs late in infection that is observed in VIDO R2s cells. Earlier studies have demonstrated that inhibition of host cell protein synthesis enhances the translation of late adenoviral mRNAs (Zhang et al., 1994), which results in an increase in the production of progeny virus in infected cells. It is possible that in VIDO R2kd cells, both viral and cellular mRNAs may compete for the limited translation factors. Alternatively, it is possible that knockdown of DDX3 may affect the translation of BAdV-3 late

protein mRNAs which contain complex 5’ untranslated regions (UTRs) (Dolph et al., 1988**).** Indeed, a recent report suggests that DDX3 specifically promotes translation of mRNAs containing complex 5’UTRs (Soto-Rifo et al., 2012). The binding of both DDX3 and pVIII to TPL of BAdV-3 and their detection in the polysome fractions containing 40S ribosomal subunits suggest that both proteins may involve in the selective translation initiation of late viral mRNAs.

In summary, we demonstrate that DDX3 is essential for efficient replication of BAdV-3 during late times post infection possibly by augmenting the efficiency of the translation of late viral mRNAs and suppression of cellular protein synthesis. Moreover, we demonstrated that the translation of TPL containing mRNAs at their 5’ ends is enhanced in the presence of DDX3 and pVIII.

8. GENERAL DISCUSSION AND CONCLUSION

Determination of biological functions of different structural and non-structural proteins of BAdV-3 is fundamental for elucidating the virus-host interaction and development of safe and efficient BAdV-3 based vaccine/antigen delivery vehicles. This work was performed to characterize the molecular properties and functions of protein pVIII of BAdV-3 which was identified as an interacting partner of the cellular protein DDX3 based on yeast II hybrid screening.

Using rabbit antisera raised against peptides representing N-terminus and C-terminus of pVIII, we detected pVIII as a 24 kDa protein starting from 12 to 24 hr post BAdV-3 infection or in pVIII transfected cells. The 24 kDa precursor form of BAdV-3 pVIII is present in immature virions, only 8 kDa cleaved portion representing "C" terminal 70 amino acids of BAdV-3 pVIII are incorporated in the mature virions. Similar reports have been made on pVIII of human adenoviruses (Chelius *et al.,* 2002; Takahashi *et al.,* 2006). This is consistent with the earlier reports that infectious virus contains only cleaved form of pVIII (Chelius *et al.,* 2002; Takahashi *et al.,* 2006) and confirms that cleavage of few structural protein by BAdV-3 protease is required for formation of mature infectious virion (Gastaldelli et al., 2008).

Localization of pVIII to the nucleus in transfected cells indicate that other viral proteins are not involved in this process suggesting that that pVIII may contain nuclear localization signal(s) involved in active transport of pVIII into the nucleus. pVIII also localizes in the cytoplasm (in a lesser amount compared to the amount that localizes in the nucleus) indicating that the protein may have multiple functions in the cytoplasm and nucleus. Proteins that are transported into the nucleus in an energy dependent mechanism use soluble nuclear transport factors like imp-α (Gorlich *et al.,* 1995; Imamoto *et al.,* 1995) or transportin (Lai et al., 2001).

Like 52K protein of BAdV-3 (Patterson et al., 2012), the nuclear transport of BAdV-3 pVIII is mediated by the Imp α/β heterodimer dependent pathway, by preferentially interacting with Impα-3, which requires ATP and RanGTP. Ran GTP controls transport receptor-substrate interactions in a compartment-specific manner between the cytoplasm and the nucleus and is required for active transport of proteins into the nucleus (Gorlich et al., 1996b). Deletion mutation analysis of pVIII suggests that the "N" terminal domain contains Impα-3 interacting domain/nuclear localization signal.

The appearance of pVIII in the form of punctate dots in BAdV-3 infected cells appears similar to the expression pattern reported for some herpesvirus essential proteins in the nucleus (de Bruyn et al., 1998; Daikoku et al., 2005) suggesting that pVIII may also be involved in replication of BAdV-3. Thus, transport of BAdV-3 pVIII to the nucleus of virus infected cells may be a requirement for the efficient replication of BAdV-3. Identification of nuclear localization signal of pVIII between amino acid 57-72 should help in determining the function of BAdV-3 pVIII in the nucleus by creating and analyzing recombinant BAdV-3 expressing the NLS deleted or point mutants of NLS of pVIII.

A number of viral-viral and viral- cellular protein-protein interactions are important to modulate various cellular functions for proper adenoviral DNA replication, assembly and release (Cuesta and Schneider, 2004; Ostapchuk and Hearing, 2008; Perez-Romero et al., 2005; Singh et al., 2005; Cuesta et al., 2000). Since viral proteins may require interaction with other cellular proteins to perform some function (Vidalain and Tangy, 2010; Friedel and Haas, 2011), we determined if pVIII interacts with any cellular protein. Moreover, using various assays, we confirmed that pVIII interacts with DDX3 in BAdV-3 infected cells. It would be interesting to explore the absolute requirements of such interactions for efficient replication of BAdV-3 by

creating and analyzing recombinant BAdV-3 expressing interacting domain deleted or point mutants of BAdV-3 pVIII.

Recent studies have suggested that DDX3, a member of the family of DEAD box protein of RNA helicases (Rocak and Linder, 2004) enhances translation initiation by interacting with different translation initiation factors (Shih et al., 2008; Lai et al., 2008; Shih et al., 2011; Lee et al., 2008). Interestingly, inhibition of cellular protein synthesis, especially during late time post infection (Dolph et al., 1988; Huang and Schneider, 1991) have suggested the involvement of one or more late adenoviral proteins in the process of inhibition of cellular capped mRNA translation. Our studies confirmed that pVIII-DDX3 interaction inhibit cap dependent translation of cellular mRNAs. Earlier studies have suggested that another late adenoviral protein 100K inhibit cellular protein synthesis by the displacement of the kinase Mnk1 from the eIF4F complex (Cuesta et al., 2004). It is possible that at different stage of replication, different viral proteins may be involved in altering the same cellular function suggesting that cellular proteins may be functionally redundant (Berk, 2005). Thus, during BAdV-3 infection pVIII and 100K may contribute to the overall reduction in cellular protein synthesis inhibition by employing different mechanisms.

Earlier studies demonstrated the involvement of adenovirus 100K protein in inhibiting cellular mRNA translation and enhancing translation of adenovirus late mRNA containing TPL. The binding of both DDX3 and pVIII to TPL of BAdV-3 and significant reduction in the titer of the virus in DDX3 knock down cells suggest both proteins may be involved in the selective translation initiation of late viral mRNAs. Thus, like adenovirus 100K protein (Cuesta et al., 2004), BAdV-3 pVIII appears to inhibit cellular mRNA translation and enhance translation of late mRNAs containing TPL. While adenovirus 100K protein enhances the late mRNA

translation by ribosome shunting mechanism (Xi et al., 2004), it remains to be determined how binding of pVIII and DDX3 enhance the translation of late mRNAs containing TPL.

Finally, we believe that our detailed molecular and functional characterization of BAdV-3 pVIII gives a very good insight to the role of the protein in the life cycle of the virus. In addition, our data suggest that the presence of the un-translated 5' TPL sequence helps in the selective translation of late BAdV-3 mRNAs in the presence of pVIII and DDX3. Thus, TPL may be used to achieve high transgene expression in BAdV-3 vector transduced cells which can be driven by the MLP promoter. This can also help in reducing the dose of the vector administered reducing the degree of toxicity associated with high dose.

On the other hand, the virus titer was lower in DDX3 knock down VIDO R2 cells. Hence, we presume that DDX3 over expressing cell lines may support improved replication of BAdV-3. If this can be proven, DDX3 over expressing cells can be used for virus production with increased titer.

9. FUTURE DIRECTIONS

The present work explored the importance of pVIII-DDX3 interaction in translation of cellular mRNAs and potential involvement in selective translation of BAdV-3 late mRNAs. Since DDX3 has also been involved in transcription, RNA splicing and induction of innate immunity, future work could focus on determining the effect of the interaction of DDX3 - pVIII in these cellular processes in BAdV-3 replication.

Secondly, the domain of pVIII interacting with DDX3 can be identified and the biological function of these interactions can be determined by constructing and analyzing the mutant BAdV-3 expressing interacting domain deleted/point mutated pVIII.

Finally, the mechanism by which the TPL and DDX3-pVIII interaction enhance the translation of late viral mRNAs could be investigated.

10. REFERENCES

Adam SA. 1999. Transport pathways of macromolecules between the nucleus and the cytoplasm. Curr Opin Cell Biol 11: 402–406.

Adam SA, Marr RS, Gerace L. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. J Cell Bio 111: 807-816.

Adam RL, Wente SR. 2013. Uncovering nuclear pore complexity with innovation. Cell152:1218-1221.

Ahola T, Kaarianinen L. 1995. Reaction in alphavirus mRNA capping: Formation of a covalent complex of non-structural protein nsP1 with 7-methyl-GMP. Proc Natl Acad Sci 92: 507-511.

Al Yacoub N, Romanowska M, Haritonova N, Foerster J. 2007. Optimized production and concentration of lentiviral vectors containing large inserts. J Gene Med 9:579–584.

Allen TD, Cronshaw JM, Bagley S, Kiseleva E, Goldberg MW. 2000. The nuclear pore complex: mediator of translocation between nucleus and cytoplasm. J Cell Sci 113: 1651-1659.

Anand KA, Singh J, Gaba A, Tikoo SK. 2014. Effect of bovine adenovirus 3 on mitochondria. Veterinary research 45: 45-53.

Anderson CW, Baum PR, Gesteland RF. 1973. Processing of adenovirus proteins. J Virol 12: 241-252.

Andreou AZ, Klostermeier D. 2013. The DEAD-box helicase eIF4A: Paradigm or the odd one out? RNA Biology 10(1): 19-32.

Ariumi Y, Kuroki M, Abe K, Dansako H, Ikeda M, Wakita T, Kato N. 2007. DDX3 DEAD box RNA helicase is required for hepatitis C virus RNA replication. J Virol 81: 13922-13926.

Arnberg N. 2012. Adenovirus receptors: implications for targeting of viral vectors. Trends pharmacol Sci 33(8): 442-448.

Ayalew LE, Gaba A, Kumar P, Tikoo SK. 2014. Conserved regions of Bovine Adenovirus-3 pVIII contain functional domains involved in nuclear localization and packaging in mature infectious virion. J Gen Virol (In press).

Bangari DS, Mittal SK. 2006. Development of nonhuman adenoviruses as vaccine vectors. Vaccine 24: 849-862.

Bartha A. 1969. Proposal for grouping of bovine adenoviruses. Acta Vet Acad Sci Hung 19 (3): 319-321.

Baxi MK, Deregt D, Robertson J, Babiuk LA, Schlapp T, Tikoo SK. 2000. Recombinant bovine adenovirus type 3 expressing bovine viral diarrhea virus glycoprotein E2 induces an immune response in cotton rats. Virology 278: 234-243.

Baxi MK, Reddy PS, Zakhartchouk AN, Idamakanti N, Pyne C, Babiuk LA, Tikoo SK. 1998. Characterization of bovine adenovirus type 3 early region 2B. Virus Genes 16: 313-316.

Baxi MK, Robertson J, Babiuk LA, Tikoo SK. 2001. Mutational analysis of early region 4 of bovine adenovirus type 3. Virology 290: 153-163.

Bayliss R, Leung SW, Baker RP, Quimby BB, Corbett AH, Stewart M. 2002. Structural basis for the interaction between NTF2 and nucleoporin FxFG repeats. EMBO J 17: 6587-6598.

Bayliss R, Littlewood T, Stewart M. 2000. Structural basis for the interaction between FxFG nucleoporin repeats and importin-beta in nuclear trafficking. Cell 102: 99-108.

Beck M, Forster F, Ecke M, Plitzko JM, Melchior F, Gerisch G, Baumeister W, Medalia O. 2004. Nuclear pore complex structure and dynamics revealed by cryoelectron tomography. Science 306: 1387-1390.

Bednenko J, Cingolani G, Gerace L. 2003. Importin β contains a COOH-terminal nucleoporin binding region important for nuclear transport. J Cell Biol 162: 391-401.

Belsham GJ, Mclnerney GM, Ross-Smith N. 2000. Foot-and-mouth disease virus 3C protease induces cleavage of translation initiation factors eIF4A and eIF4G within infected cells. J Virol 74(1): 272-280.

Beltz GA, Flint SJ. 1979. Inhibition of HeLa cell protein synthesis during adenovirus infection. Restriction of cellular messenger RNA sequences to the nucleus. J Mol Biol 131:353-373.

Berk AJ. 2005. Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. Oncogene. 24:7673-7685.

Berk AJ. 2007. Adenoviridae: The viruses and their replication. In: Fields Virology, 5th ed. D.

Berthelot K, Muldoon MRajkowitsch L, Hughes J, McCarthy JE. 2004. Dynamics and processivity of 40S ribosome scanning on mRNA in yeast. Mol Microbial 51:987-1001.

Blanche F, Monegier B, Faucher D, Duchesne M, Audhuy F, Barbot A, Bouvier S, Daude G, Dubois H, Guillemin T, Maton L. 2001. Polypeptide composition of an adenovirus type 5 used in cancer gene therapy. J. Chromatography A 921: 39-48.

Bol GM, Raman V, van der Groep P, Vermeulen JF, Patel AH, van der Wall E, van Diest PJ. 2013. Expression of the RNA helicase DDX3 and the hypoxia response in breast cancer. PLoS One 8 (5): e63548.

Botlagunta M, Vesuna F, Mironchik Y, Raman A, Lisok A, Winnard P Jr, Mukadam S, Van Diest P, Chen JH, Farabaugh P, Patel AH, Raman V. 2008. Oncogenic role of DDX3 in breast cancer biogenesis. Oncogene 27:3912-3922.

Breiman A, Grandvaux N, Lin R, Ottone C, Akira S, Yoneyama M, Fujita T, Hiscott J, Meurs EF. 2005. Inhibition of RIG-I dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKϵ. J Virol 79 (7): 3969-3978.

Brierley I. 1995. Ribosomal frameshifting on viral RNAs. J Gen Virol 76: 1885-1892.

Brierley I, Jenner AJ, Inglis S C 1992. Mutational analysis of the 'slippery sequence' component of a coronavirus ribosomal frameshifting signal. J Mol Biol 227: 463-479.

Brierley I, Rolley NJ, Jenner AJ, Inglis SC. 1991. Mutational analysis of the RNA pseudoknot component of a coronavirus ribosomal frameshifting signal. J Mol Biol 220: 889-902.

Brook M, McCracken L, Reddington JP, Lu ZL, Morrice NA, Gray NK. 2012. The multifunctional poly(A)-binding protein (PABP) 1 is subject to extensive dynamic post-translational modification, which molecular modeling suggesats plays an important role in co-ordinating its activities. J Bio chem 441: 803-812.

Burgert HGZ, Ruzsics S, Obermeier A, Hilgendorf M, Windheim M, Elsing A. 2002. Subversion of host defense mechanisms by adenoviruses. Curr Top Microbiol Immunol 269: 273-318.

Burgui I, Yanguez E, Sonenberg N, Nieto A. 2007. Influenza virus mRNA translation revisited: is the eIF4E cap-binding factor required for viral mRNA translation? J Virol 81: 12427–12438.

Bushell M, Sarnow P. 2002. Hijacking the translation apparatus by RNA viruses. J. cell Biol 158: 395-399.

Chahar HS, Chen S, Manjunath N. 2012. P-body components LSM1, GW182, DDx3, DDX6 and XRN1 are recruited to WNV replication sites and positively regulate viral replication. Virology. 436 (1): 1-7.

Chang PC, Chi CW, Chau GY, Li FY, Tsai YH, Wu JC, Wu Lee YH. 2006. DDX3, a DEAD box RNA helicase, is deregulated in hepatitis virus-associated hepatocellular carcinoma and is involved in cell growth control. Oncogene 25: 1991–2003.

Chao CH, Chen CM, Cheng PL, Shih JW, Tsou AP, Lee YH. 2006. DDX3, a DEAD box RNA helicase with tumor growth suppressive property and transcriptional regulation activity of the p21waf1/cip1 promoter, is a candidate tumor suppressor. Cancer Res 66: 6579-6588.

Chartier C, Degryse E, Gantzer M, Dieterle A, Pavirani A, Mehtali M. 1996. Efficient generation of recombinant adenovirus vectors by homologous recombination in escherichia coli. J Virol 70: 4805-4810.

Chelius D, Huhmer AF, Sgieh CH, Lehmberg E, Traina JA, Slattery TK, Pungor EJr. 2002. Analysis of the adenovirus-5 proteome by liquid chromatography and tandem mass spectrometry methods. J Proteome Res 1: 501-513.

Chen X, Chamorro M, Lee SI, Shen LX, Hines JV, Tinoco IJr, Varmus HE. 1995. Structural and functional studies of retroviral RNA pseudoknots involved in ribosomal frameshifting: nucleotides at the junction of the two stems are important for efficient ribosomal frameshifting. EMBO J 14: 842-852.

Chenik M, Chebli K, Blondel D. 1995. Translation initiation at alternate in-frame AUG codons in the rabies virus phosphoprotein mRNA is mediated by a ribosomal leaky scanning mechanism. J Virol 69(2): 707–712.

Chook YM, Blobel G. 1999. Structure of the nuclear transport complex karyopherin-β2-RanGppNHp. Nature 399: 230-237.

Chook YM, Suel KE. 2011. Nuclear import by karyopherin-βs: recognition and inhibition. Biochemica et Biophysica Acta 1813:1593-1606.

Christophe D, Christophe-Hobertus C, Pichon B. 2000. Nuclear targeting of proteins: how many different signals? Cell Signal 12: 337-341.

Chuang RY, Weaver PL, Liu Z, Chang TH. 1997. Requirement of the DEAD-Box protein ded1p for messenger RNA translation. Science 275: 1468–1471.

Cingolani G, Petosa C, Weis K, Müller CW. 1999. Structure of importin-beta bound to the IBB domain of importin-alpha. Nature 399: 221-229.

Coenjaerts FE, van Oosterhout JA, van der Vliet PC. 1994. The Oct-1 POU domain stimulates adenovirus DNA replication by a direct interaction between the viral precursor terminal protein-DNA polymerase complex and the POU homeodomain. EMBO J 13: 5401-5409.

Cong P, Shuman S. 1995. Mutational analysis of mRNA capping enzyme identifies amino acids involved in GTP binding, enzyme-guanylate formation, and GMP transfer to RNA. Mol Cell Biol 15: 6222–6231.

Connor JH, Lyles DS. 2002. Vesicular stomatitis virus infection alters the eIF4F translation initiation complex and causes the dephosphorylation of the eIF4E binding protein 4E-BP1. J Virol 76 (20): 10177-10187.

Cook A, Bono F, Jinek M, Conti E. 2007. Structural biology of nucleocytoplasmic transport.Annu Rev Biochem 76: 647-671.

Cordin O, Banroques J, Tanner NK, Linder P. 2006. The DEAD-box protein family of RNA helicases. Gene 367:17-37.

Cortes P, Ye ZS, Baltimore D. 1994. RAG-1 interacts with the repeated amino acid motif of the human homologue of the yeast protein SRP1. Proc Nat Acad Sci 91:7633-7637.

Crawford-Miksza L, Schnurr DP. 1996. Analysis of 15 adenovirus hexon proteins reveals thelocation and structure of seven hypervariable regions containing serotype-specific residues. J Virol 70: 1836-1844.

Cuesta R, Xi Q, Schneider RJ. 2000. Adenovirus specific translationby selective disassembly of cap-initiation complex. EMBO J 19: 3465–3474.

Cuesta R, Xi Q, Schneider RJ. 2004. Structural basis for competitive inhibition of eIF4G-Mnk1interaction by the adenovirus 100-kilodalton protein. J Virol 78: 7707-7716.

Daikoku T, Kudoh A, Fujita M, SugayaY, Isomura H, Shirata N, Tsurumi T. 2005. Architecture of replication compartments formed during Epstein-Barr virus lytic replication. J Virol 79**:** 3409–3418.

Dang CV, Lee WM. 1988. Identification of the human c-myc protein nuclear translocation signal. Mol Cell Biol 8(10): 4048-4054.

Darbyshire JH, Dawson PS, Lamont PH, Ostler DC, Pereira HG. 1965. A new adenovirus serotype of bovine origin. J Comp Pathol 75: 327-330.

Darbyshire JH, Jennings AR, Dawson PS, Lamont PH, Omar AR. 1966. The pathogenesis and pathology of infection in calves with a strain of bovine adenovirus type 3. Res Vet Sci 7:81-93.

Daughenbaugh KF, Fraser CS, Hershey JWB, Hardy ME. 2003. The genome-linked protein VPg of the Norwalk virus binds eIF3, suggesting its role in translation initiation complex recruitment. EMBO J 22: 2852–2859.

Davis LI. 1995. The nuclear pore compex. Annu. Rev. Biochem 64: 865-896.

Davison AJ, Benko M, Harrach B. 2003. Genetic content and evolution of adenoviruses. J Gen Virol 84: 2895-2908.

Dayton AI. 2004. Within you, without you: HIV-1 Rev and RNA export. Commentary. Retrovirology 1:35.

de Breyne S, Simonet V, Pelet T, Curran J. 2003. Identification of a cis-acting element required for shunt mediated translational initiation of the Sendai virus Y proteins. Nucleic Acids Res 31:608-618.

de Breyne S, Yo Y, Unbehaun A, Pestova TV, Hellen CUT. 2009. Direct functional interaction of initiation factor eIF4G with type 1 internal ribosome entry sites. Proc Natl Acad Sci 106(23): 9197-9202.

de Bruyn Kops A, Uprichard SL, Chen M, Knipe DM. 1998. Comparison of the intranuclear distributions of herpes simplex virus proteins involved in various viral functions. Virology 252: 162–178.

de Jong RN, van der Vliet PC, Brenkman AB. 2003. Adenovirus DNA replication: protein priming, jumping back and the role of the DNA binding protein DBP. Curr Top Microbiol Immunol 272: 187-211.

De la Cruz J, Iost I, Kressler D, Linder P. 1997. The p20 and Ded1 proteins have antagonistic roles in eIF4E dependent translation in Sachromyces cerevisiae. Proc Natl Acad Sci 94:5201-5206.

Decroly E, Ferron F, Lescar J, Canard B. 2012. Conventional and unconvential mechanisms for capping viral mRNAs. Nature Reviews Microbiology 10: 51-65.

Depping R, Steinhoff A, Schindler SG, Friedrich B, Fagerlund R, Metzen E, Hartmann E, Köhler M. 2008. Nuclear translocation of hypoxia-inducible factors (HIFs): involvement of the classical importin alpha/beta pathway. Biochim Biophys Acta 1783: 394-404.

Dingwall C, Laskey RA. 1991. Nuclear targeting sequences- a consensus ? trends Biochem. Science 16: 478-481.

Dinman JD. 2012. Mechanisms and implications of programmed translational frameshifting. Wiley Interdiscip Rev RNA.3(5):661-73.

Dinman JD. 2006. Programmed ribosomal frameshifting goes beyond viruses: Microbe wash DC 1(11): 521-527.

Diouri M, Kewani-Amineh H, Geoghegan KF, Weber JM. 1996. Cleavage efficiency by adenovirus protease is site-dependent. J Biol Chem 271(51):32511-32514.

Ditton HJ, Zimmer J, Kamp C, Rajpert-De Meyts E, Vogt PH. 2004. The *AZFa* gene *DBY (DDX3Y)* is widely transcribed but the protein is limited to the male germ cells by translation control. Human Molecular genetics, 19: 2333-2341.

Dolph PJ, Racaniello V, Villamarin A, Palladino F, Schneider RJ. 1988. The adenovirus tripartite leader may eliminate the requirement for cap-binding protein complex during translation initiation. J Virol 62: 2059-2066.

Du E, Tikoo SK. 2010. Efficient replication and generation of recombinant bovine adenovirus-3 in nonbovine cotton rat lung cells expressing I-SceI endonuclease. The J of Gen Med 12:840-847.

Eckmann CR, Rammelt C, Wahle E. 2010. Control of poly (A) tail length. Wiley Interdiscip Rev RNA 2(3): 348-361.

Edgil D, Polacek C, Harris E. 2006. Dengue virus utilizes a novel strategy for translation initiation when cap-dependent translation is inhibited. J Virol 80: 2976-2986.

Fadden P, Haystead TA, Lawrence JC. 1997. Identification of Phosphorylation SitesIn the Translational Regu}ator, PHAS-I, That Are ControlledBy Insulin and Rapamycin InRat Adipocytes. J. Biol. Chem 272: 10240-10247.

Feigenblum D, Schneider RJ. 1993. Modification of eukaryotic initiation factor by 4F during infection by influenza virus. J Virol 67(6): 3027-3035.

Feldher CM, Kallenbach E, Schultz N. 1984. Movement of karyophilic protein through the nuclear pores of oocytes. J Cell Bio 99: 2216-2222.

Ferrari R, Pellegrini M, Horwitz GA, Xie W, Berk AJ, Kurdistani SK. 2008. Epigenetic reprogramming by adenovirus e1a. Science. 2321:1086-1088.

Ferron F, Decroly E, Selisko B, Canard B. 2012. The viral RNA capping machinery as a target for aniviral drugs. Antiviral Res 96(1): 21-31.

Fischer PM. 2009. Cap in hand: Targeting eIF4E. Cell cycle 8(16): 2535-2541.

Fitzgerald KD, Semler BL. 2009. Bridging IRES elements in mRNAs to the eukaryotic translation apparatus. Biochim Biophys Acta 1789(9–10): 518–528.

Fornerold M, Vandeursen J, Vanbaal S, Reynolds A, Davis D, Murti KG, Fransen J, Grosveld G. 1997. The human homologue of yeast Crm1 is in a dynamic subcomplex with Can/Nup214 and a novel nuclear pore component Nup88. EMBO J 16: 807-816.

Forwood JK, Harley V, Jans DA. 2001. Nuclear import of Creb and AP-1 transcription factors requires importin-β 1 and Ran but is independent of importin-alpha. Biochemistry 40: 5208-5217.

Forwood JK, Lange A, Zachariae U, Marfori M, Preast C, Grubmuller H, Stewart M, Corbett AH, Kobe B. 2010. Quantitative structural analysis of importin beta flexibility: paradigm for solenoid protein structures. Structure 18(9): 1171-1183.

Fried H, Kutay U. 2003. Nucleocytoplasmic transport: taking an inventory. Cell Mol Life Sci 60:1659-1688.

Friedel CC, Haas J. 2011. Virus-host interactomes and global models of virus-infected cells. Trends in Microbiology. 19(10):501-508.

Friedrich B, Quensel C, Sommer T, Hartmann E, Kohler M. 2006. Nuclear localization signal and protein context both mediate importin alpha specificity of nuclear import substrates. Mol Cell Biol 26: 8697-8709.

Frisch SM, Mymryk JS. 2002. Adenovirus-5 E1A: paradox and paradigm. Nat Rev Mol Cell Biol. 3:441-452

Fuller-Pace FV. 2006. DexD/H box RNA helicase: multifunctional proteins with important roles in transcriptional regulation. Nucleic acid research. 34:4206-15.

Furuichi Y, Shatkin A J. 2000. Viral and cellular mRNA capping: past and prospects.Adv. Vir Res 55: 135–184.

Futterer J, Kiss-Laszlo Z, Hohn T. 1993. Nonlinear ribosome migration on cauliflower mosaic virus 35S RNA. Cell 73(4): 789–802.

Futterer J, Potrykus I, Bao Y, Li L, Burns, TM, Hull R, Hohn T. 1996. Position dependent ATT initiation during plant pararetrovirus Rice Tungro Bacilliform Virus translation. J.Virol. 70:2999-3010.

Gastaldelli M, Imelli N, Boucke K, Amstutz B, Meier O, Greber UF. 2008. Infectious adenovirus type 2 transport through early not late endosomes. Traffic 9: 2265-2278.

Geissler R, Golbik RP, Behrens S. 2012. The DEAD-box helicase DDX3 supports the assembly of functional 80S ribosomes. Nucleic Acid Res 1-14.

Giedroc DP, Cornish PV. 2009. Frameshifting RNA pseudokbots: Structure and mechanism. Virus research 139: 193-208.

Gingras A, Raught B, Sonenberg N. 1999. eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 68:913–963.

Gingras A, Raught B, Sonenberg N. 2001. Regulation of translation initiation by FRAP/mTOR. Genes & Development 15:807-826.

Goldfarb DS, Corbett AH, Mason DA, Harreman MT, Adam SA. 2004. Importin alpha: a multipurpose nuclear-transport receptor. Trends Cell Biol 14: 505-514.

Goodfellow I, Chaudhry Y, Gioldasi I, Gerondopoulos A, Natoni A, Labrie L, Laliberté JF, Roberts L. 2005 Calicivirus translation initiation requires an interaction between VPg and eIF 4 E. EMBO 6(10):968-72

Goodwin DJ, Whitehouse A. 2001. A γ-2 herpesvirus nucleocytoplasmic shuttle protein interacts with importin α-1 and α-5. The j of bio chem 276 (23): 19905-19912.

Görlich D, Henklein P, Laskey RA, Hartmann E. 1996a. A 41 amino acid motif in importin-alpha confers binding to importin-beta and hence transit into the nucleus. EMBO J 15: 1810-1817.

Görlich D, Kraft R, Kostka S, Vogel F, Hartmann E, Laskey RA, Mattaj IW, Izaurralde E. 1996b. Importin provides a link between nuclear protein import and U snRNA export. Cell 87: 21-32.

Gorlich D, Mattaj IW. 1996. Nucleocytoplasmic transport. Science 271: 1513-1518.

Görlich D, Panté N, Kutay U, Aebi U, Bischoff FR. 1996c. Identification of different roles for RanGDP and RanGTP in nuclear protein import. EMBO J 15: 5584-5594.

Gorlich D. 1997. Nuclear protein import. Curr Opin Cell Biol 9:412-419.

Gorlich D, Mattaj IW. 1996. Nucleocytoplasmic transport. Science 271: 1513-1518.

Gorlich D, Kostka S, Kraft R, Laskey RA, Hartmann E, Prehn S. 1995. Two different subunits of importin cooperate to recognize nuclea localization signals and bind them to the nuclear envelope. Curr Biol 5(4): 383-392.

Gräble M, Hearing P. 1992. Cis and trans requirements for the selective packaging of adenovirus type 5 DNA. J Virol 66: 723-731.

Gross T, Siepmann A, Sturm D, Windgassen M, Scarcelli JJ, Seedorf M, Cole CN, Krebber H. 2007. The DEAD-box RNA helicase Dbp5 functions in translation termination. Science 315(5812): 646-649.

Gross DJ, Kleiman FE. 2013. Poly (A) binding proteins: are they all created equal? Wiley Interdiscip Rev RNA 4(2): 167-179.

Gu L, Tsuji T, Jarboui MA, Yeo GP, Sheeshy N, Hall WW, Gautier VW. 2011. Intermolecular masking of the HIV-1 Rev NLS by the cellular protein HIC: Novel insights into the regulation of Rev nuclear import. Retrovirology 8:17-29.

Gupta S, Mangel WF, McGrath WJ, Perek JL, Lee DW, Takamoto K, Chance MR. 2004. DNA binding provides a molecular strap activating the adenovirus proteinase. Mol Cell Proteomics 3: 950-959.

Hanover JA, Love DC, Prinz WA. 2009. Calmodulin-driven nuclear entry: Trigger for sex determination and terminal differentiation. J Biol Chem 12593-12597.

Harrach B. 2000. Reptile adenoviruses in cattle? Acta Vet Hung 48: 485-490.

Hellen CU, Sarnow P. 2001. Internal ribosome entry sites in eukaryotic mRNA molecules. Genes Dev 15(13): 1593-1612.

Hellen CU. 2009. IRES-induced conformational changes in the ribosome and the mechanism of translation initiation by internal ribosomal entry. Biochim Biophys Acta 1789(9–10): 558–570.

Herold A, Truant R, Wiegand H, Cullen BR. 1998. Determination of the functional domain organization of the importin alpha nuclear import factor. J Cell Biol 143: 309-318.

Hershey JWB, Merrick WC. 2000. The pathway and mechanism of initiation of protein synthesis. Translational control of gene expression. Sonenberg N, Hershey JWB, Mathews MB. Cold spring harbor laboratory press. 33-88.

Higashimoto Y, Yamagata Y, Itoh H. 2006. Complex effect of adenovirus early region proteins on innate immune system. Inflamm Allergy Drug Targets. 5:229-237.

Hilleman MR, Werner JH. 1954. Recovery of new agent from patients with acute respiratory illness. Proc Soc Exp Biol Med 85: 183-188.

Hilliker A, Gao Z, Jankowsky E, Parker R. 2011. The DEAD box protein Ded1 modulates translation by the formation and resolution of an eIF4F-mRNA complex. Molecular Cell 43:962-972.

Hindley CE, Lawrence FJ, Matthews DA. 2007. A role for transportin in the nuclear import of adenovirus core proteins and DNA. Traffic 8: 1313-1322.

Hinnebusch AG. 2000. Mechanism and regulation of initiator Methionyl-tRNA binding to ribosomes. In: Translational control of gene expression. Alan G, Hershey, JWB, Mathews MB. Cold spring harbor laboratory press. 185-243.

Hodge LD, Scharff MD. 1969. Effect of adenovirus on host cell DNA synthesis in synchronized cells. Virology 37: 554-564.

Holcik M, Sonenberg N. 2005. Translational control in stress and apoptosis. Nat Rev Mol Cell Biol 6: 318–327.

Hong SS, Szolajska E, Schoehn G, Franqueville L, Myhre S, Lindholm L, Ruigrok RWH, Boulanger P, Chroboczek J. 2005. The 100K-chaperone protein from adenovirus serotype 2 (Subgroup C) assists in trimerization and nuclear localization of hexons from subgroups C and B adenoviruses. J Mol Biol 352: 125-138.

Horwitz MS. 2004. Function of adenovirus E3 proteins and their interactions with immunoregulatory cell proteins. J Gene Med 6 (Suppl 1): S172-183.

Hu SL, Hays WW, Potts DE. 1984. Sequence homology between bovine and human adenoviruses. J Virol 49: 604-608.

Huang JT, Schneider RJ. 1991. Adenovirus inhibition of cellular protein synthesis involves inactivation of cap-binding protein. Cell 65: 271-280.

Idamakanti N, Reddy PS, Babiuk LA, Tikoo SK. 1999. Transcription mapping and characterization of 284R and 121R proteins produced from early region 3 of bovine adenovirus type 3. Virology 256: 351-359.

Ilkow CS, Mancinelli V, Beatch MD, Hobman TC. 2008. Rubella virus capsid protein interacts with poly (A)-binding protein and inhibits translation. J Virol 82(9): 4284-4294.

Imamoto N, Shimamoto T, Takao T, Tachibana T, Kose S, Matsubae M, Sekimoto T, Shimonishi Y, Yoneda Y. 1995. In vivo evidence for involvement of a 58 kDa component of nuclear pore targeting complex in nuclear protein import. EMBO J 14: 3617-3626.

Imataka H, Gradi A, Sonenberg N. 1998. A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. EMBO J 17: 7480–7489.

Imataka H, Sonenberg N. 1997. Human eukaryotic translation initiation factor 4G (eIF4G) possesses two separate and independent binding sites for eIF4A. Mol Cell Biol 17: 6940–6947.

International Committee on Taxonomy of Viruses, King AMQ. 2012. Virus taxonomy :classification and nomenclature of viruses : ninth report of the International Committee on Taxonomy of Viruses. Academic Press, London.

Ishaq M, Hu J, Wu X, Fu Q, Yang Y, Liu Q, Guo D. 2008. Knockdown of cellular RNA helicaseDDX3 by short hairpin RNAs suppresses HIV-1 viral replication without inducing apoptosis. Mol Biotechnol 39: 231-238.

Jackson RJ, Hellen CUT, Pestova TV. 2010. The mechanism of eukaryotic translation initiation and principles of its regulation. Nature reviews/Molecular Cell Biology 10:113-127.

Jacobson A. 1996. Poly(A) metabolism and translation: The closed-loop model. In *Translational control* (ed. N. Sonenberg), pp.451–480. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Jäkel S, Görlich D. 1998. Importin beta, transportin, RanBP5 and RanBP7 mediate nuclear import of ribosomal proteins in mammalian cells. EMBO J 17: 4491-4502.

Jang SK, Krausslich HG, Nicklin MJ, Duke GM, Palmenberg AC, Wimmer E. 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J Virol 62:2636–2643.

Jia Y, Polunovsky V, Bitterman PB, Wagner CR. 2012. Cap-dependent translation initiation factor eIF4E: An emerging anticancer drug target. Medicinal research reviews 32(4):786-814.

Jivotovskaya AV, Valášek L, Hinnebusch AG, Nielsen KH. 2006. Eukaryotic Translation Initiation Factor 3 (eIF3) and eIF2 Can Promote mRNA Binding to 40S Subunits Independently of eIF4G in Yeast. Mol Cell Biol 26 (24): 1355–1372

Kahms M, Huve J, Wesselmann R, Farr JC, Baumgartel V, Peters R. 2011. Lighting up the nuclear pore complex. European J Cell Biol 90: 751-758.

Kahvejian A, Svitkin YV, Sukarieh R, M'Boutchou M, Sonenberg N. 2005. Mammalian poly (A)-binding protein is a eukaryotic translation initiation factor, which acts via multiple mechanisms. Genes and Development 19 104-113.

Katahira J, Strasser K, Podtelejnikov A, Mann M, Jung JU, Hurt E.1999. The Mex67p-mediated nuclear mRNA export pathway is conserved from yeast to human. EMBO J 18:2593–2609.

Kataoka N, Bachorik JL, Dreyfuss G. 1999. Transportin-SR, a nuclear import receptor for SR proteins. J Cell Biol 145: 1145-1152.

Katharine SU, Powers MA, Forbes DJ. 1997. Nuclear export receptors: From importin to exportin. Cell 90: 967-970.

Kieff E. 1996. Epstein–Barr virus and its replication. In Fields, B.N., Knipe, D.M., Howley, P.M., Chanock, R.M., Melnick, J.L., Monath, T., Roizman, B. & Straus, S.E. (eds), Virology. *Lippincott–Raven Publishers*, 2343–2396.

Kieft JS. 2008. Viral IRES RNA structures and ribosome interactions. Trends Biochem Sci 33(6): 274–283.

Kohler M, Gorlich D, Hartmann E, Franke J. 2001. Adenoviral E1A protein nuclear import is preferentially mediated by importin alpha3 in vitro. Virology 289: 186-191.

Kohler M, Speck C, Christian M, Bischoff FR, Prehn S, Haller H, Gorlich D, Hartmann E. 1999. Evidence for distinct substrate specificities of importin α family member in nuclear protein import. Molecular and cellular biology 19(11): 7782-7791.

Kohtz JD, Jamison SF, Will CL, Zuo P, Luhrmann R, GarciaBlanco MA, Manley JL 1994. Protein-protein interactions and 59-splice-site recognition in mammalian mRNA precursors. Nature 368, 119-124.

Kollmus H, Honigman A, Panet A, Hauser H. 1994. The sequences of and distance between two cis-acting signals determine the efficiency of ribosomal frameshifting in human immunodeficiency virus type 1 and human T-cell leukemia virus type II in vivo. J Virology 68: 6087-6091.

Kolupaeva VG, Lomakin IB, Pestova TV, Hellen CU. 2003. Eukaryotic initiation factors 4G and 4A mediate conformational changes downstream of the initiation codon of the encephalomyocarditis virus internal ribosomal entry site. Mol Cell Biol. 2003 Jan;23(2):687-98.

Kozak M. 1991a. An analysis of vertebrate mRNA sequences: intimations of translational control. J Cell Biol 115: 887–903.

Kozak M. 1991b. 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J Biol Chem 266: 19867–19870.

Kulshreshtha V. 2009. Molecular Characterization of 33K Protein of Bovine Adenovirus Type 3. A Thesis submitted to University of Saskatchewan.

Kulshreshtha V, Tikoo SK. 2008. Interaction of bovine adenovirus-3 33K protein with other viral proteins. Virology 381(1): 29-35.

Kulshreshtha V. 2009. Molecular characterization of 33K protein of bovine adenovirus type 3. PhD Thesis, University of Saskatchewan, Saskatoon. Pp 132.

Kulshreshtha V, Babiuk LA, Tikoo SK. 2004. Role of bovine adenovirus-3 33K protein in viral replication. Virology 323: 59-69.

Kwong AD, Rao BG, Jeang KT. 2005. Viral and cellular RNA helicases as antiviral targets. Nat Rev Drug Discov 10:845-53.

Laakkonen P, Hyvönen M, Peränen J, Kääriäinen L.1994. Expression of Semliki Forest Virus Nsp1-Specific Methyltransferase in Insect Cells and in Escherichia-Coli. Journal of Virology 68: 7418-7425.

Lai M, Chang W, Shieh S, Tarn W. 2010. DDX3 regulates cell growth through translational control of cyclin E1. Molecular and cellular biology 30(22): 5444-5453.

Lai M, Lee YW, Tarn W. 2008. The DEAD box RNA helicase DDX3 associates with export messenger ribonucleoproteins as well as tip-associated protein and participates in translational control. Molecular biology of the cell 16: 3847-3858.

Lai MC, Lin RI, Tarn WY. 2001. Transportin-SR2 mediates nuclear import of phosphorylated SR proteins. Proc Natl Acad Sci 98: 10154-10159.

Lange A, Mills RE, Lange CJ, Stewart M, Devine SE, Corbett AH. 2007. Classical nuclear localization signals: definition, function and interaction with importin α. The J of Biol Chem 282 (8): 5101-5105.

Lee SJ, Matsuura Y, Liu SM, Stewart M. 2005. Structural basis for nuclear import complex dissociation by RanGTP. Nature 435: 693-696.

Lee TW, Blair GE, Matthews DA. 2003. Adenovirus core protein VII contains distinct sequences that mediate targeting to the nucleus and nucleolus, and colocalization with human chromosomes. J Gen Virol 84: 3423-3428.

Lee C, Dias AP, Jedrychowski M, Patel AH, Hsu JL, Reed R. 2008. Human DDX3 functions in translation and interacts with the translation initiation factor eIF3. Nucleic acids research. 36(14): 4708-4718.

Lehmkuhl HD, Hobbs LA. 2008. Serologic and hexon phylogenetic analysis of ruminant adenoviruses. Arch virology 153: 891-897.

Lehmkuhl HD, Smith MH, Dierks RE. 1975. A bovine adenovirus type 3: isolation, characterization, and experimental infection in calves. Arch virology 48: 39-46.

Li J, Wang JT, Whelan SP. 2006. A unique strategy for mRNA cap methylation used by vesicular stomatitis virus. Proc Natl Acad Sci U S A 103: 8493-8498.

Lim RYH, Aebi U, Fahrenkrog B. 2008. Towards reconciling structure and function in the nuclear pore complex. Histochem Cell Biol 129: 105-116.

Linder P, Jankowsky E. 2011. From unwinding to clamping-the DEAD box RNA helicase family. Nature reviews Mol Cell Biol 12: 505-516

Linne T, Philipson L. 1980. Further characterization of the phosphate moiety of the adenovirus type 2 DNA-binding protein. Eur J Biochem. 103:259-270.

Lisowski P, Pierzchala M, Goscik J, Pareek CS, Zwierzchowski L. 2008. Evaluation of reference genes for studies of gene expression in the bovine liver, kidney pitutary and thyroid. J Appl Genet 49(4): 367-372.

Liu F, Green MR. 1994. Promoter targeting by adenovirus E1a through interaction with different cellular DNA-binding domains. Nature 368: 520-525.

Liu J, Henao-Mejia J, Liu H, Zhao Y, He JJ. 2011. Translational regulation of HIV-1 replication by HIV-1 Rev cellular cofactors Sam68, eIF5A, hRIP, and DDX3. J Neuroimmune Pharmacol 6: 308-321.

Liu GQ, Babiss LE, Volkert FC, Young CS, Ginsberg HS. 1985. A thermolabile mutant of adenovirus 5 resulting from a substitution mutation in the protein eight gene. J Virol 53: 920-925.

Liu H, Jin L, Koh SB, Atanasov I, Schein S, Wu L, Zhou ZH. 2010. Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. Science 329: 1038-1043.

Liu Y, Vellekamp G, Chen G, Mirza UA, Wylie D, Twarowska B, Tang JT, Porter FW, Wang S, Nagabhushan TL, Pramanik BN. 2003. Proteomic study of recombinant adenovirus 5 encoding human p53 by matrix-assisted laser desorption/ionization mass spectrometry in combination with database search. Int. J. Mass Spectrom 226: 55-69.

Lopez-Lastra M, Ramdohr P, Letelier A, Vallejos M, Vera-Otarola J, Valiente-Echeverria F. 2010. Translation initiation of viral mRNAs. Reviews in Medical Virology 20:177-195.

Lopez-Lastra M, Rivas A, Barria MI. 2005. Protein synthesis in eukaryotes: the growing biological relevance of cap-dependent translation initiation. Biological research 38: 121-146.

Lyons RH, Ferguson BQ, Rosenberg M. 1987. Pentapeptide nuclear localization signal in adenovirus E1a. Mol Cell Biol 7(7): 2451-1456.

Mader S, Lee H, Pause A, Sonenberg N. 1995. The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4γ and the translational repressors 4E-binding proteins. Mol Cell Biol 15: 4990-4997

Makadiya N. 2013. Functional characterization of 100K protein of bovine adenovirus type 3. PhD Thesis, University of Saskatchewan.

Malim MH, Hauber J, Le SY, Maizel JV, Cullen BR. 1989. The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA. Nature 338: 254–257.

Mamiya N, Worman HJ. 1999. Hepatits C virus core protein binds to a DEAD box RNA helicase. J Bio Chem 274:15751-6.

Marfori M, Mynott A, Ellis JJ, mehdi AM, Saunders NFW, Curmi PM, Forwood JK, Boden M, Kobe B. 2011. Molecular basis for specificity of nuclear import and prediction of nuclear localization. Biochem Biophys Acta 1813(9): 1562-1577

Marsden S, Nardelli M, Linder P, McCarthy JE. 2006. Unwinding single RNA molecules using helicases involved in eukaryotic translation initiation. J Mol Biol 361(2): 327-335.

Martin CS. 2012. Latest insights on adenovirus structure and assembly. Viruses 4(12): 847-877

Martinez-Palomo A, Granboulan N. 1967. Electron microscopy of adenovirus 12 replication. II. High-resolution autoradiography of infected KB cells labeled with tritiated thymidine. J Virol 1: 1010-1018.

Martinez-Palomo A, Le Buis J, Bernhard W. 1967. Electron microscopy of adenovirus 12 replication. 1. Fine structural changes in the nucleus of infected KB cells. J Virol 1: 817-829.

McConnell MJ, Imperiale MJ. 2004. Biology of adenovirus and its use as a vector for gene therapy. Human gene therapy 15: 1022-1033.

Melen K, Fagerlund R, Franke J, Kohler M, Kinnunen L, Julkunen I. 2003. Importin alpha nuclear localization signal binding sites for STAT1, STAT2, and influenza A virus nucleoprotein. J Biol Chem 278: 28193–28200.

Methot N, Song MS, Sonenberg N. 1996. A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. Mol. Cell. Biol 16:5328–5334.

Mi S, Stollar V. 1991. Expression of Sindbis virus nsP1 and methyltransferase activity in Escherichia coli. Virology 184: 423-427.

Mitrousis G, Olia AS, Walker-Kopp, N, Cingolani G. 2008. Molecular basis for the recognition of snurportin 1 by importin beta. J Biol Chem 283: 7877-7884.

Mittal SK, Middleton DM, Tikoo SK, Babiuk LA. 1995. Pathogenesis and immunogenicity of bovine adenovirus type 3 in cotton rats (Sigmodon hispidus). Virology 213: 131-139.

Mittal SK, Tikoo SK, Van Donkersgoed J, Beskorwayne T, Godson DL, Babiuk LA. 1999. Experimental inoculation of heifers with bovine adenovirus type 3. Can J Vet Res 63(2): 153-156.

Mittal SK, Prevec L, Babiuk LA, Graham FL. 1992. Sequence analysis of bovine adenovirus type 3 early region 3 and fibre protein genes. J Gen Virol 73 (Pt 12): 3295-3300.

Moore JD, Yang J, Truant R, kornbluth S. 1999. Nuclear import of Cdk/cyclin complexes: identification of distinct mechanisms for import of Cdk2/cyclin E and Cdc2/cyclin B1. J cell Biol 144: 213-224.

Moore MS. 1998. Ran and nuclear transport. J Biol Chem 273: 22857-22860.

Murphy AM, Grdzelishvili VZ. 2009. Identificationof Sendai virus L protein aminoacid residues affecting viral mRNA Cap methylation. J Virol 83(4): 1669-1681.

Nakielny S, Dreyfuss G. 1998. Import and export of the nuclear protein import receptor transportin by a mechanism independent of GTP hydrolysis. Curr Biol 8: 89-95.

Nemerow GR, Pache L, Reddy V, Stewart PL. 2009. Insights into adenovirus host cell interactions from structural studies. Virology 384: 380-388.

Nemerow GR, Stewart PL, Reddy VL. 2012. Structure of human adenovirus. Curr Opin Virol 2(2): 115-121.

Newmeyer DD, Forbes DJ. 1988. Nuclear import can be separated into distinct steps: *in-vitro* nuclear poe binding and translocation. Cell 52: 641-653.

Ogino T, Banerjee AK. 2007. Unconventional mechanism of mRNA capping by the RNA-dependent RNA polymerase of vesicular stomatitis virus. Mol Cell 25: 85–97.

Orlando JS, Ornelles DA. 1999. An arginine facd amphibatic alpha helix is required for adenovirus type 5 E4orf6 function. J Virol 73: 4600-4610.

O'Shea C, Klupsch K, Choi S, Bagus B, Soria C, Shen J, McCormick F, Stokoe D. 2005. Adenoviral Proteins Mimic nutrient/growth Signals to activate the mTOR Pathway for Viral Replication. EMBO J 24: 1211-1221.

Oshiumi H, Ikeda M, Matsumoto M, Watanabe A, Takeuchi O, Akira S, Kato N, Shimotohno K, Seya T. 2010. Hepatitis C virus core protein abrogates the DDX3 function that enhances IPS-1 mediated IFN-beta induction. Plos One 5 (12): e14258.

Ostapchuk P, Almond M, Hearing P. 2011. Characterization of Empty Adenovirus Particles Assembled in the Absence of a Functional Adenovirus IVa2 Protein. J. Virol 85: 5524-5531.

Owsianka AM, Patel AH. 1999. Hepatitis C virus core protein interacts with a human DEAD box protein DDX3. Virology 257:330-340.

Paine PL, Moore LC, Horowitz SB. 1975. Nuclear envelope permeability. Nature 254: 109-114.

Parrish S, Resch W, Moss B. 2007. Vaccinia virus D10 protein has mRNA decapping activity, providing a mechanism for control of host and viral gene expression. Proc Natl Acad Sci 104: 2139-2144

Parsyan A, Svitkin Y, Shahbazian D, Gkogkas C, Lasko P, Merrick WC, Sonenberg N. 2011. mRNA helicases: the tacticians of translational control. Nat Rev Mol Cell Biol 12: 235–245.

Paterson CP. 2010. Molecular characterization of 52K protein of bovine adenovirus type 3. PhD Thesis, University of Saskatchewan, Saskatoon.

Paterson CP, Ayalew LE, Tikoo SK. 2012. Mapping of nuclear import signal and importinα3 binding regions of 52K protein of bovine adenovirus-3. Virology 432: 63-72.

Pause A, Belsham GJ, Gingras AC, Donze O, Lin TA, Lawrence JC, Sonenberg N. 1994. Insulin-dependentstimulation ofprotein synthesis by phosphorylation of a regulalor of 5'-cap function. Nature 371: 762-767.

Pelletier J, Sonenberg N. 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature 334(6180): 320–325.

Pelletier J, Flynn ME, Kaplan G, Racaniello V, Sonenberg N. 1988. Mutational analysis of upstream AUG codons of poliovirus RNA. J Virol 62:4486–4492.

Pemberton LF, Paschal BM. 2005. Mechanisms of receptor mediated nuclear import and nuclear export. Traffic 6:187-198.

Pestova TV, Hellen CU, Shatsky IN. 1996a. Canonical eukaryotic initiation factors determine initiation of translation by internal ribosomal entry. Mol Cell Biol 16:6859–6869.

Pestova TV, Shatsky IN, Hellen CU. 1996b. Functional dissection of eukaryotic initiation factor 4F: The 4A subunit and the central domain of the 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. Mol Cell Biol 16:6870–6878.

Pestova TV, Lorsch J, Hellen CUT. 2007. in Translational Control in Biology and Medicine, edsMathews M, Sonenberg N, Hershey J (Cold Spring Harbor Lab Press, Cold Spring Harbor, NY), pp87–120.

Pestova TV, Kolupaeva VG. 2002. The roles of individual eukaryotic translation initiation factors in ribosomal scanning and initiation codon selection. Genes Dev 16:2906–2922.

Phillips DM, Raskas HJ. 1972. Ultrastructural changes in KB cultures infected with adenovirus type 2. Virology 48: 156-169.

Phizicky EM, Fields S. 1995. Protein-Protein Interactions: Methods for Detection and Analysis. Microbiological reviews 59:94-123.

Piron M, Vende P, Cohen J, Poncet D. 1998. Rotavirus RNA-binding protein NSP3 interacts with eIF4GI and evicts the poly(A) binding protein from eIF4F. EMBO J 17:5811–5821.

Poulin F, Gingras AC, Olsen H, Chevalier S, Sonenberg N. 1998. 4E-BP3, a new member of the eukaryotic initiation factor 4E-binding protein family. J Biol Chem 273:14002-14007.

Puvion-Dutilleul F, Bachellerie JP, Visa N, Puvion E. 1994. Rearrangements of intranuclear structures involved in RNA processing in response to adenovirus infection. J Cell Sci 107 (6): 1457-1468.

Rajendran RR, Nye AC, Fraser J. 2003. Regulation of nuclear receptor transcriptional activity by a novel DEAD box RNA helicase (DP97). J Biol Chem 278: 4628-4638.

Rau M, Ohlmann T, Morley SJ, Pain VM. 1996. A reevalulation of the cap-binding protein, eIF4E, as a rate-limiting factor for initiation of translation in reticulocyte lysate. J Biol Chem 271: 8983-8990

Raught B, Gingras AC. 1999. eIF4E activity is regulated at multiple levels. Int J Biochem Cell Biol 31:43–57.

Reddy PS, Chen Y, Idamakanti N, Pyne C, Babiuk LA, Tikoo SK. 1999a. Characterization of early region 1 and pIX of bovine adenovirus-3. Virology 253: 299-308.

Reddy PS, Idamakanti N, Chen Y, Whale T, Babiuk LA, Mehtali M, Tikoo SK. 1999b. Replication-defective bovine adenovirus type 3 as an expression vector. J Virol 73: 9137-9144.

Reddy PS, Idamakanti N, Zakhartchouk AN, Baxi MK, Lee JB, Pyne C, Babiuk LA, Tikoo SK. 1998. Nucleotide sequence, genome organization, and transcription map of bovine adenovirus type 3. J Virol 72: 1394-1402.

Reddy VS, Natchiar SK, Stewart PL, Nemerow GR. 2010. Crystal Structure of Human Adenovirus at 3.5 Å Resolution. Science 329, 1071-1075.

Remm M, Remm A, Ustav M. 1999. Human papillomavirus type 18 E1 protein is translated from polycistronic mRNA by a discontinuous scanning mechanism. J Virol 73: 3062-3070.

Richardson WD, Mills AD, Dilworth SM, Laskey RA, Dingwall C. 1988. Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell 52: 665-66.

Rocak S, Linder P. 2004. DEAD box proteins: The driving forces behind RNA metabolism. Nature Reviews. Mol Cell Bio 5(3): 232-241.

Rohn K, Prusas C, Monreal G, Hess M. 1997. Identification and characterization of penton base and pVIII protein of egg drop syndrome virus. Virus Research 47: 59-65.

Ron D, Harding HB. 2007. eIF2a phosphorylation in cellular stress response and disease. In ‘‘Translational Control in Biology and Medicine.’’ (M. B. Mathews, N. Sonenberg, and J. W. B. Hershey, Eds.), pp. 345–368. Cold Spring Harbor Laboratory Press, New York.

Rong L, Livingstone M, Sukarieh R, Petroulakis E, Gingras AC, Crosby K, Smith B, Polakiewicz RD, Pelletier J, Ferraiuolo MA,Sonenberg N. 2008. Control of eIF4E cellular localization by eIF4E-binding proteins, 4E-BPs. RNA 14:1318–1327.

Rout MP, Aitchison JD, Suprapto A, Hjertaas K, Zhao Y, Chait, BT. 2000. The yeast nuclear pore complex: Composition, architecture, and transport mechanism. J Cell Biol 148: 635-651.

Rowe WP, Huebner RJ, Gilmore LK, Parrott RH, Ward TG. 1953. Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc Soc Exp Biol Med 84: 570-573.

Rowlands AG, Panniers R, Henshaw EC. 1988. The catalytic mechanism of guanine nucleotide exchange factor action and competitive inhibition by phosphorylated eukaryotic initiation factor 2. J Biol Chem 263: 5526–5533.

Roy S, Vandenberghe LH, Kryazhimskiy S, Grant R, Calcedo R, Yuan X, Keough M, Sandhu A, Wang Q, Madina-Jaszek A, Plotkin JB, Wilson JM. 2009. Isolation and characterization of adenoviruses persistently shed from the gastro-intestinal tract of non-human primates. PLOS pathogens. 5(7):e1000503.

Ruigrok RW, Barge A, Mittal SK, Jacrot B. 1994. The fibre of bovine adenovirus type 3 is very long but bent. J Gen Virol 75: 2069-2073.

Russell WC, Kemp GD. 1995. Role of adenoviral structural components in the regulation of adenovirus infection. Curr Top Microbiol Immunol 199: 81-98.

Russell WC. 2000. Update on adenovirus and its vectors. J Gen Virol 81:2573-2604.

Russel WC.2009. Adenoviruses: update on structure and function. J Gen Virol 90(1):1-20.

Sarnow P, Cevallos RC, Jan E.2005. Takeover of host ribosomes by divergent IRES elements. Biochem Soc Trans 33(6): 1479–1482.

Sato S, Fukasawa M, Yamakawa Y, Natsume T, Suzuki T, Shoji I, Aizaki H, Miyamura T, Nishijima M. 2006. Proteomic profiling of lipid droplet proteins in hepatoma cell lines expressing hepatitis C virus core protein. J. Biochem. 139: 921–930.

Schaecher SR, Mackenzie JM, Pekosz A. 2007. The ORF7b protein of severe acute respiratory syndrome coronavirus (SARS-CoV) is expressed in virus-infected cells and incorporated into SARS-CoV particles. J Virol 81(2): 718–731.

Schaley JE, Polonskaia M, Hearing P. 2005. The adenovirus E4-6/7 protein directs nuclear localization of E2F-4 via an arginine-rich motif. J Virol 79: 2301-2308.

Schneider RJ, Mohr I. 2003. Translation initiation and viral tricks. Trends Biochem Sci 28(3): 130-136.

Schroder M. 2010. Human DEAD box protein 3 has multiple functions in gene regulation and cell cycle control and is a prime target for viral manipulation. Biochem Pharmacol 79:297-306.

Schroder M, Baran M, Bowie AG. 2008. Viral targeting of DEAD box protein 3 reveals its role in TBK1/IKK epsilon mediated IRF activation. EMBO J 27:2147-2157.

Schwartz S, Felber BK, Fenyo EM, Pavlakis GN. 1990. Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. J Virol 64(11): 5448–5456.

Schwartz S, Felber BK, Fenyo EM, Pavlakis GN. 1990. Env and Vpu proteins of human immunodeficiency virus type 1 are produced from multiple bicistronic mRNAs. J Virol 64: 5448–5456.

Sen N, Cao F, Tavis JE. 2004. Translation of duck hepatits B virus reverse transcriptase by ribosome shunting. J Virol 78: 11751-11757.

Shenk T. 2001. Adenoviridae: The viruses and their replication. In: Fields Virology, 4th ed. D. Knipe and P. Howley (Ed.). Philadelphia: Lippincott Williams & Wilkins. pp.2265-2300.

Shih JW, Tsai TY, Chao CH, Wu Lee YH. 2008. Candidate tumor suppressor DDX3 RNA helicase specifically represses cap-dependent translation by acting as an eIF4E inhibitory protein. Oncogene 27: 700–714.

Shih JW, Wang WT, Tsai TY, Kuo CY, Li HK, Wu Lee YH. 2012. Critical roles of RNA helicase DDX3 and its interactions with eIF4E/PABP1 in stress granule assembly and stress response. Biochem J. 441: 119–129.

Shinagawa M, Iida Y, Matsuda A, Tsukiyama T, Sato G. 1987. Phylogenetic relationships between adenoviruses as inferred from nucleotide sequences of inverted terminal repeats. Gene 55: 85-93.

Silverman E, Edwards-Gilbert G, Lin R. 2003. DExD/H-box proteins and their partners: helping RNA helicases unwind. Virology 312: 1-16.

Singh M, Shmulevitz M, Tikoo SK. 2005. A newly identified interaction between IVa2 and pVIII proteins during porcine adenovirus type 3 infection. Virology 336, 60-69.

Siomi H, Dreyfuss G. 1995. A nuclear localization domain in the hnRNP A1 protein. J Cell Biol 129: 551-560.

Smyth JA, Benkö M, Moffett DA, Harrach B. 1996. Bovine adenovirus type 10 identified in fatal cases of adenovirus-associated enteric disease in cattle by in situ hybridization. J Clin Microbiol 34: 1270-1274.

Sokabe M, Fraser CS, Hershey JWB. 2011. The human translation initiation multi-factor complex promotes methionyl-tRNAi binding to the 40S ribosomal subunit. Nucleic acids research 1-9

Sonenberg N, Hinnebusch AG. 2009. Regulation of translation initiationin eukaryotes: mechanisms and biological targets. Cell 136(4): 731-745.

Sorokin AV, Kim ER, Ovchinnikov LP. 2007. Nucleocytoplasmic transport of proteins. Biochemistry (Moscow) 72(13): 1439-1457.

Soto-Rifo R, Rubilar PS, Limousin T, de Breyne S, Decimo D, Ohlmann T. 2012. DEAD-box protein DDX3 associates with eIF4F to promote translation of selected mRNAs. EMBO J. 31: 3745-3756.

Souza1 APD, Haut L, Reyes-Sandoval A, Pinto1 AR. 2005. Recombinant viruses as vaccines against viral diseases. Brazilian Journal of Medical and Biological Research 38: 509- 522.

Stacey SN, Jordan D, Williamson AJ, Brown M, Coote JH, Arrand JR. 2000. Leaky scanning is the predominant mechanism for translation of human papillomavirus type 16 E7 oncoprotein from E6/E7 bicistronic mRNA. J Virol 74: 7284–7297.

Stewart M. 2007. Molecular mechanism of the nuclear protein import cycle. Nature reviews/Mol cell Biol 8:195-208.

Stoneley M, Willis AE. 2004. Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. Oncogene 23(18): 3200–3207.

Sweitzer TD, Hanover JA. 1996. Calmodulin activates nuclear protein import: a link between signal transduction and nuclear transport. Proc Natl Acad Sci USA 93: 14574-14579.

Tachibana T, Hieda M, Miyamoto Y, Kose S, Imamoto N, Yoneda Y. 2000. Recycling of importin alpha from the nucleus is suppressed by loss of RCC1 function in living mammalian cells. Cell Struct Funct 25, 115-123.

Takahashi E, Cohen SL, Tsai PK, Sweeney JA. 2006. Quantitation of adenovirus type 5 empty camsids. Anal Biochem 349: 208-217.

Tarn WY, Chang TH. 2009. The current understanding of Ded1p/DDX3 homologs from yeast to human. RNA Biol 6(1):17-20.

Tatsis N, Ertl HCJ. 2004. Adenoviruses as Vaccine Vectors. Molecular Therapy 10: 616-629.

Tauber B, Dobner T. 2001. Adenovirus early E4 genes in viral oncogenesis. Oncogene 20:7847-7854.

Theerthagiri G, Eisenhardt N, Schwarz H, Antonin W. 2010. The nucleoporin Nup188 controls passage of membrane proteins across the nuclear pore complex. The Journal of Cell Biology 189 (7): 1129-1142.

Tollefson AE, Hermiston TW, Wold WS. 1998. Preparation and titration of CsCl-banded adenovirus stock. Adenovirus Methods and Protocols 1-9.

Tran EJ, Wente SR. 2006. Dynamic nuclear pore complexes: life on the edge. Cell 125: 1041-1053.

Tremblay ML, Dery CV, Talbot BG, Weber J. 1983. *In-vitro* cleavage specificity of the adenovirus type 2 proteinase. Biochimica et biophysica acta 743, 239-245.

Truant R, Cullen BR. 1999. The arginine-rich domains present in human immunodeficiency virustype 1 Tat and Rev function as direct importin beta-dependent nuclear localization signals. Mol Cell Biol 19: 1210-1217.

Turnell AS, Mymryk JS. 2006. Roles for the coactivators CBP and p300 and the APC/CE3 ubiquitin ligase in E1A-dependent cell transformation. Br J Cancer. 95:555-560.

van Breukelen B, Brenkman AB, Holthuizen PE, van der Vliet PC. 2003. Adenovirus Type 5 DNA Binding Protein Stimulates Binding of DNA Polymerase to the Replication Origin. J. Virol 77: 915-922.

Van Der Kelen K, Bayaert R, Inze D, De Veylder L. 2009. Translational control of eukaryotic gene expression. Critical reviews Biochem & Mol Biol 44(4):143-168.

Vashist S, Urena L, Chaudhry Y, Goodfellow I. 2012. Identificatio of RNA-protein interaction networks involved in the norovirus life cycle. J Virol 86:11977-11990.

Vasu SK, Forbes DJ. 2001. Nuclear pores and nuclear assembly. Curr Opin Cell Biol 13: 363-375.

Vellekamp G, Porter FW, Sutjipto S, Cutler C, Bondoc L, liu Y, Wylie D, Cannon-Carlson S, Tang JT, Frei A, Voloch M, Zhuang S. 2001. Empty capsids in column-purified recombinant adenovirus preparations. Human gene therapy 12: 1923-1936.

Vidalain P, Tangy F. 2010. Virus-host interactions in RNA viruses. Microbes and Infection 1-10.

Wagstaff KM, Jans DA. 2009. Importins and beyond: non-conventional nuclear transport mechanisms. Traffic 10:1188-1198.

Walsh D, Mohr I. 2011. Viral subversion of the host protein synthesis machinery. Nat Rev Microbiol 9(12): 860-875.

Wang H, Ryu W. 2010. Hepatitis B virus polymerase blocks pattern recognition receptor signaling via interaction with DDX3: implications for immune evasion. PLoS pathog 6(7): e1000986.

Wang H, Kim S, Ryu WS. 2009. DDX3 DEAD box RNA helicase inhibits hepatitis B virus reverse transcription by incorporations into nucleocapsids. J Virol 83:5815-5824.

Wang K, Pearson GD. 1985. Adenovirus sequences required for replication in vivo.Nucleic Acids Res 13:5173-5187.

Wang P, Palese P, O'Neill RE. 1997. The NPI-/ NPI-3 (karyopherin *a*) binding site on the influenza A virus nucleoprotein NP is a nonconventional nuclear localization signal. J Virol 71, 1850-1856.

Watanabe M, Yanagisawa J, Kitagawa H, Takeyama K, Ogawa S, Arao Y, Suzawa M, Kobayashi Y, Yano T, Yoshikawa H, Masuhiro Y, Kato S. 2001. A sub-family of RNA-binding DEAD-box proteins acts as estrogen α coactivator through the N-terminal activation domain (AF-1) with an RNA coactivator. SRA. EMBO J 20:1341-1352.

Webster A, Russell WC, Kemp GD. 1989. Characterization of the adenovirus proteinase; development and use of a specific peptide assay. J of Gen Virol 70: 3215-3223.

Weeks DL, Jones NC. 1985. Adenovirus E3-early promoter: sequences required for activation by E1A. Nucleic Acids Res 13: 5389-5402.

Weill L, Belloc E, Bava F, Mendez R. 2012. Translational control by changes in poly (A) tail length: recycling mRNAs. Nature Structural & Mol Biol 9(6): 577-585.

Weitzman MD, Ornelles DA. 2005. Inactivating intracellular antiviral responses during adenovirus infection. Oncogene 24: 7686-7696.

Welch K, Franke J, Kohler M, Macara IG. 1999. RanBP3 contains an unusual nuclear localization signal that is imported preferentially by importin-α3. Mol Cell Biol 19: 8400–8411.

Wells SA, Hillner PE, Vale RD, Sachs AB. 1998. Circularization of mRNA by eukaryotic translation initiation factors. Molecular Cell 2:135-140.

Wente SR, Rout MP. 2010. The nuclear pore complex an nuclear transport. Cold spring harb perspect boil 2:000562.

White E. 1996. Life, Death, and the Pursuit of Apoptosis. Genes Dev 10: 1-15.

White E. 2001. Regulation of the cell cycle and apoptosis by the oncogenes of adenovirus. Oncogene 20: 7836-7846.

Willcocks MM, Carter MJ, Roberts LO. 2004. Cleavage of eukaryotic initiation factor eIF4G and inhibition of host-cell protein synthesis during feline calicivirus infection. J Gen Virol 85: 1125-1130.

Wodrich H, Cassany A, D'Angelo MA, Guan T, Nemerow G, Gerace L. 2006. Adenovirus core protein pVII is translocated into the nucleus by multiple import receptor pathways. J Virol 80: 9608-9618.

Wodrich H, Guan T, Cingolani G, Von Seggern D, Nemerow G, Gerace L. 2003. Switch from capsid protein import to adenovirus assembly by cleavage of nuclear transport signals. EMBO J 22: 6245-6255.

Wold WS, Tollefson AE, Hermiston TW. 1995. E3 transcription unit of adenovirus. Curr Top Microbiol Immunol 199: 237-274.

Wong CM, McFall ER, Burns JK, Parks RJ. 2013. The Role of Chromatin in Adenoviral Vector Function. Viruses 5: 1500-1515.

Wu Q, Chen Y, Kulshreshtha V, Tikoo S. 2004. Characterization and nuclear localization of the fiber protein encoded by the late region 7 of bovine adenovirus type 3. Arch virology 149: 1783-1799.

Wu JY, Maniatis T. 1993. Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. Cell 75: 1061-1070.

Wu Q, Tikoo SK. 2004. Altered tropism of recombinant bovine adenovirus type-3 expressing chimeric fiber. Virus Res 99: 9-15.

Xi Q, Cuesta R, Schneider RJ. 2004.Tethering of eIF4G to adenoviral mRNAs by viral 100k protein drives ribosome shunting. Genes Dev 18(16): 1997-2009.

Xi Q, Cuesta R, Schneider RJ. 2005. Regulation of translation by ribosome shunting through phosphotyrosine- dependent coupling of adenovirus protein 100k to viral mRNAs. J Virol 79(9): 5676-5683.

Xing L, Tikoo SK. 2006. E1A promoter of bovine adenovirus type 3. J Gen Virol 87: 3539-3544.

Xing L, Tikoo SK. 2007. Bovine adenovirus-3 E1A coding region contain cis-acting DNA packaging motifs. Virus Res 130: 315-320.

Xing L, Zhang L, Van Kessel J, Tikoo SK. 2003. Identification of cis-acting sequences required for selective packaging of bovine adenovirus type 3 DNA. J Gen Virol 84: 2947-2956.

Xu D, Farmer A, Chook YM. 2010. Recognition of nuclear targeting signals by karyopherin-β proteins. Curr opinion in Struc Biol 20:782-790.

Xu K, Zheng BJ, Zeng R, Lu W, Lin YP, Xue L, Li L, Yang LL, Xu C, Dai J, Wang F, Li Q, Dong QX, Yang RF, Wu JR, Sun B. 2009. Severe acute respiratory syndrome coronavirus accessory protein 9b is a virion-associated protein. Virology 388(2):279-285.

Yakhnin AV, Yakhnin H, Babitzke P. 2012. Gel mobility shift assays to detect protein-RNA interactions. Methods Mol Biol 905: 201-2011.

Yang Y, Hussain S, Wang H, Ke M, Guo D. 2009. Translational control of the subgenomic RNAs of severe acute respiratory syndrome Coronavirus. Virus Genes 39(1): 10-18.

Yedavalli VSRK, Neuveut C, Chi Y, Kleiman L, Jeang K. 2004. Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function. Cell 119:381-392.

Yoneda Y, Imamaoto-Sonobe N, Yamaizumi M, Uchida T. 1987. Reversible inhibition of protein import into the nucleus by wheat germ agglutinin injected into cultured cells. Exp Cell Res 173: 586-595.

Yoneyama M, Kikuchi M. Natsukawa T, Shinobu N, Imaizumi T, Miyagishi M, Taira K, Akira S, Fujita T. 2004. The RNA helicase RIG-I has an essential function in double stranded RNA induced innate anti viral responses. Nat Immunol 5:730-737

You LR, Chen CM, Yeh TS, Tsai TY, Mai RT, Lin CH, Lee, YH. 1999. Hepatitis C virus core protein interacts with cellular putative RNA helicase. J Virol 73:2841-2853.

Yu S, Chen J, Wu M, Chen H, Kato N, Yuan Z. 2010. Hepatitis B virus polymerase inhibits RIG-I and toll-like receptor 3-mediated beta interferon induction in human hepatocytes through interference with interferon regulatory factor 3 activation and dampening of the interaction between TBK1/IKKε and DDX3. J of Gen Virol 91:2080-2090.

Yueh A, Schneider RJ. 1996. Selective translation initiation by ribosome jumping in adenovirus-infected and heat shocked cells. Genes Dev **10**: 1557-1567.

Yueh A, Schneider RJ. 2000. Translation by ribosome shunting on adenovirus and hsp70 mRNAs facilitated by complementarity to 18S rRNA. Genes Dev 14(4):414-421.

Zakhartchouk A, Connors W, van Kessel A, Tikoo SK. 2004. Bovine adenovirus type 3 containing heterologous protein in the C-terminus of minor capsid protein IX. Virology 320: 291-300.

Zakhartchouk AN, Godson DL, Babiuk LA, Tikoo SK. 2001. 121R protein from the E3 region of bovine adenovirus-3 inhibits cytolysis of mouse cells by human tumor necrosis factor. Intervirology 44: 29-35.

Zakhartchouk AN, Pyne C, Mutwiri GK, Papp Z, Baca-Estrada ME, Griebel P, Babiuk LA, Tikoo SK. 1999. Mucosal immunization of calves with recombinant bovine adenovirus-3: induction of protective immunity to bovine herpesvirus-1. J Gen Virol 80 (5): 1263-1269.

Zakhartchouk AN, Reddy PS, Baxi M, Baca-Estrada ME, Mehtali M, Babiuk LA, Tikoo SK. 1998. Construction and characterization of E3-deleted bovine adenovirus type 3 expressing fulllength and truncated form of bovine herpesvirus type 1 glycoprotein gD. Virology 250: 220-229.

Zakhartchouk, A.N., Viswanathan, S., Mahony, J.B., and Babiuk, L.A. (2005) Severe acute respiratory syndrome coronavirus nucleocapsid protein expressed by an adenovirus vector is phosphorylated and immunogenic in mice. *J. Gen. Virol*. **86**:211-215

Zhai ZH, Nickerson JA, Krochmalnic G, Penman S (1987) Alterations in nuclear matrix structure after adenovirus infection. J Virol 61: 1007-18.

Zhang Y, Feigenblum D, Schneider RJ. 1994. A late adenovirus factor induces eIF-4E dephosphorylation and inhibition of cell protein synthesis. J Virol 68:7040-7050

Zheng B, Mittal SK, Graham FL, Prevec L. 1994. The E1 sequence of bovine adenovirus type 3 and complementation of human adenovirus type 5 E1A function in bovine cells. Virus Res 31:163-186.

Zheng BJ, Graham FL, Prevec L. 1999. Transcription units of E1a, E1b and pIX regions of bovine adenovirus type 3. J Gen Virol 80: 1735-1742.

Zhou Y, Pyne C, Tikoo SK. 2001a. Determination of bovine adenovirus-3 titer based on immunohistochemical detection of DNA binding protein in infected cells. J Virol Methods 94: 147-153.

Zhou Y, Reddy PS, Babiuk LA, Tikoo SK. 2001b. Bovine adenovirus type 3 E1B (small) protein is essential for growth in bovine fibroblast cells. Virology 288: 264-274.

Zhou Y, Tikoo SK. 2001. Analysis of early region 1 of porcine adenovirus type 3. Virology 291: 68-76.